"Changes in protein during differentiation".

by

Mavis Hilary Tulett

Thesis presented for the degree of Doctor of Philosophy
of the University of Edinburgh in the Faculty of Science.

February 1967

INDEX.

I. INTRODUCTION

(i) Background.

The morphological and biochemical changes which take place in plant cells during their passage from a meristematic to a mature state, have been the object of much study in recent years. Compared with meristematic cells, mature cells are different in size, shape and metabolism.

As a result of the work with roots by Brown (1963) and his co-workers, it is now well established that the growth in size of cells during the early stages of development is not a passive process involving only the absorption of water into the vacuole, but is a complex process involving changes in the metabolic state of the cell. Expansion is a growth process accompanied by an increase in dry matter. If the metabolic activity of cells is inhibited, expansion is also affected.

Brown and Robinson (1955) put forward the hypothesis that the growth of a cell is determined by a development which involves a sequence of metabolic changes. These changes include an apparent decrease in the capacity for division. It was thought that mature cells no longer possessed the potential for meristematic activity, but Steward, et. al. (1958) has shown that mature, carrot cells when cultured and given the necessary conditions (growth factors, etc.) can be induced to recommence division. The cells subsequently produce a callus from which a complete new plant can be obtained, The mature cell is therefore not limited to the role it plays in a differentiated system, but has as great a potential as a meristematic cell. The progression from a meristematic to a non-meristematic state must be due either to a change in the environmental factors, or to an inherent capacity of the cell to change its metabolic state. Since all cells in the root have the same genetic potential, the sequence

of metabolic changes must involve changes in the relative activity of the genes.

Many aspects of the changes which take place during differentiation have been studied. During development there is a 20-30-fold increase in the volume of the cell. The accompanying increase in dry weight which reaches a maximum at 9.4 mm (Brown and Broadbent, 1956), is accounted for by increases in carbohydrate material, (Jensen, 1955), proteins (Brown and Sutcliffe, 1950; Brown and Broadbent, 1951; Morgan and Reith, 1954, 1961; Cherry and Hageman, 1961), and nucleic acids (Holmes et. al. 1955; Jensen, 1955, 1958; Heyes, 1960; Lyndon, 1962).

It has also been found that there are increases in the amounts of subcellular particles. Lund, Vatter and Hanson, (1958) found an increase in the material sedimenting between 800 x g and 20,000 x g which suggests a synthesis of mitochondria, and Loening, (1961) found an increase in a membranous microsomal fraction concomitant with a decrease in the number of free ribosomes which suggested that during differentiation ribosomes become attached to membranes.

Changes in the cell wall have been reported by Jensen and Ashton (1960). Cell wall formation is a necessary condition for expansion. Root segments grown on a medium lacking in sugar cannot synthesise wall material, and in these cells, expansion is limited.

There are changes in the proportions of two RNA fractions (Heyes, 1960; Bucknall and Sutcliffe,1965). Heyes found that the changing base composition of RNA during cell expansion was due to changes in the relative proportions of two RNA fractions.

The importance of protein synthesis in growth has been shown by Blank and Frey-Wyssling, (1944), and by Brachet, (1954) who found a

correlation between the two. Although it was known that the total amount of protein was changing, until 1960 there was no information about the magnitude of the changes going on within the protein content, though indications of changes had been shown by the results of enzyme studies, studies on the amino acid composition of proteins (Morgan, and Keith 1954), and on the relative amounts of two protein fractions (Dorner, Kahn and Wildman, 1952).

The changing activity of many enzymes during root cell development involves an increase in activity to approximately 8 mm from the apex, followed by a decrease in activity in mature cells. Reports of changes in enzyme activity during development have been published by Robinson, (1952); Robinson and Brown, (1954); Brown and Robinson, (1955); Robinson, (1956); Robinson and Cartwright, (1958); Cook, (1959); Mertz, (1961); and Vaughan, (1965). Any particular enzyme, however, represents only a very small proportion of the total protein, and it is possible that an increase in the activity of an enzyme represents not an increase in its production, but only an activation of enzyme molecules already present in the meristematic cell.

In 1960, Wright, using serological techniques, showed that in oat coleoptiles of increasing age, there are changes in the proportions of serologically distinct proteins. Although this method scans a great number of the proteins present, it is limited to those proteins which are antigenic, and Wright considered it possible that there are some non-antigenic proteins in the cells.

(ii) Aim.

The aim of this study was to determine (1) the magnitude of the quantitative changes which take place in the total protein of the cell

during differentiation, (2) whether any new proteins are formed during differentiation, and (3) to investigate the sites and control of protein synthesis.

(iii) Tissue.

The ideal system for studying cell development would be to choose a single cell and follow its fate as it leaves the meristematic state and embarks on a course of differentiation. This, however, is not possible because the biochemical methods used involve destroying the cell. An alternative method is to study different cells at the different stages of development and in this way follow the progress of a representative cell through differentiation.

The tissue used for the present study was the pea seedling root. This tissue was chosen because of the large amount of work which has already been done on it in this laboratory, and because of the ease with which quantities of cells at different stages of differentiation might be obtained. These are obtained by cutting the root transversely at increasing distances from the apex (into serial segments). Results are attributed to representative cells, and in general do not take into account the fact that each segment contains epidermal, cortical and stelar cells. It has been shown by Bucknall and Sutcliffe, (1965), who compared the nucleic acid content of cells in the inner stele, outer stele and cortex, that there are differences between the cells from these tissues. The possibility that some of my results are due to a change in the proportions of the different types of cells within a segment will be discussed.

There is some doubt as to the origin of the cells midway between the apex and the base of the root. The number of cells in the embryonic radicle is, according to Lyndon (unpublished) only a few thousand short of the number in a 3.5 cm root. (This is the length used in the present study.)

This suggests that only the cells in the very tip of a 3.5 cm root have been produced since germination, and that the rest have been present in the root since its formation in the embryo. It might be expected that a sharp dividing line should exist between the old cells and the cells which have been recently produced, but a longitudinal section shows no discontinuity of this sort. It appears from the anatomy that there is a developmental sequence which extends from the meristem to the base of the root and biochemical experiments suggest that there is also a succession of metabolic states at least over the first 10 millimetres. Cells in this region can thus be described as being at different stages of differentiation and differences in the protein content of these cells attributed to changes in protein during differentiation.

(iv) Method chosen.

To study the overall changes which take place in proteins during differentiation, it was necessary to use a method by which the proteins could be fractionated as far as possible, and thus be easily scanned. For technical reasons the study had to be limited to the soluble proteins, however these do represent about half the total protein of the cell. They were extracted from pea root segments by homogenizing the tissue in a suitable medium and removing the cell debris and subcellular particles by centrifugation. If the homogenizing medium is carefully chosen it is possible to maintain the integrity of most of the cell particles e.g. mitochondria and nuclei, during the extraction procedure so that the supernatant after the centrifugation contains only the soluble proteins of the cytoplasm.

The method chosen for fractionating these proteins was electrophoresis on polyacrylamide gel (Raymond and Weintraub, 1959; Ornstein, 1961).

Since this work was started, Steward, Lyndon and Barber have published a paper (1965) describing the changes which they find occurring during development of pea roots, and more recently, Morris (1966) has described the changes taking place during the development of the same organ, though his results were rather different from those of Steward et. al.. In both cases the method used was polyacrylamide gel electrophresis, and a comparison of results obtained by these workers with my results will be found in the discussion.

(v) Protein fractionation.

Protein chemistry up until 1945 was mainly concerned with the identification of amino acids present in hydrolysates of proteins obtained from different sources, but there were some attempts made to separate whole proteins. In 1937 Tiselius described his new instrument for separating protein by the technique of moving boundary electrophoresis. This was in principle a U-tube, the arms of which were connected to electrode compartments. The technique had first been used by Picton and Linder (1897) to separate some colloids, and was adapted for proteins by Tiselius. Although proteins could be separated by moving boundary electrophoresis, there was never complete separation because of the 'dead volume' which remained in the bend of the U-tube. Because of the difficulty of recovering proteins after separation, this method was not really successful as a means of fractionation. It was used mainly to determine the mobilities of different proteins.

The method of moving boundary electrophoresis had several technical difficulties. The system was very unstable. In an attempt to overcome this and to eliminate disturbances, attempts were made to separate proteins in columns which contained a packing material, e.g. ground glass

wool (Coolidge, 1939). In such media it was possible to get complete separation of proteins into zones (i.e. fractionation of proteins) which was a considerable advantage and eliminated boundary anomalies.

The use of gels as a supporting medium for electrophoresis was first introduced by Consden Gordon and Martin in 1946. They used silica gel for separating amino acids and peptides. Agar gel was later compared with silica (Gordon, Keil and Šebesta, 1949) and found to have better properties: in silica gel, electro-osmosis was a serious complication and to prevent diffusion a high potential gradient had to be applied.

Filter paper, as a supporting medium for electrophoresis was introduced in 1951 (Kunkel and Tiselius), but the resolution of components was not very good. An improvement of this type of fractionation was devised by Kohn (1958), who used cellulose acetate membrane.

In 1952, Kunkel and Slater compared columns packed with glass beads, glasspowders, sands and resins with another gel, this time starch gel, and found it to be considerably better as a supporting medium. This method was improved by Smithies (1955) who used this technique for fractionating serum proteins. This remained the best supporting medium for electrophoresis until the introduction of polyacrylamide gel in 1959 by Ornstein and Davis, and independently by Raymond and Weintraub. The resolution of proteins achieved and the number of components obtained in starch and polyacrylamide gels was far superior to any previous method.

The methods of protein fractionation described, all involve the method of electrophoresis. There were, however, other methods, mainly chromatographic. A range of supporting media were used for chromatography: ion exchange resins (Boardman and Partridge, 1955), cellulose ion exchange (Peterson and Sober, 1956; Sober et. al. 1956) and calcium phosphate (Tiselius et. al., 1956).

Separation of proteins may involve resolving just two or a few proteins, but protein fractionation involves resolving all the molecular species present in a mixture of proteins. Besides the methods of electrophoresis and chromatography described, attempts were made to fractionate proteins by carefully controlled addition of ammonium sulphate (Derrien, 1952; Steyne-Parve and van den Hout, 1963). With this method it should be possible to fractionate proteins of any range of molecular weights. Fractionation procedures involving gels are somewhat limited in the range of molecular weights for which they can be used. This is because the mobility of the proteins in gels depends not only on their net charge, but also on the molecular weight, very large molecules being unable to enter the pores of the gel. It will be seen that polyacrylamide has the advantage that the pore size can be adjusted by varying the concentration of monomers. In this way very large molecules can be separated. It should be possible to fractionate a protein mixture containing proteins of very different molecular weights by using a gradient of gel concentrations.

There is another technique for the separation, and possibly the identification of proteins and that is immunological analysis. For this method, introduced in 1946 by Oudin, and developed by Ouchterlony (1948), the antibodies of particular proteins are allowed to react with the antigens in a gel. Agar, starch and polyacrylamide gels have all been used for this technique. The antigen and antisera may be placed in wells in the gel and allowed to diffuse towards each other so that precipitin bands are formed where they meet, or the antigens may be separated by electrophoresis first, then the antisera applied in grooves cut along the length of the gel. This technique can also be applied to paper and cellulose acetate membrane.

(vi) Polyacrylamide gel electrophoresis

Polyacrylamide was recommended to Ornstein by Oster, who had been studying the polymerization of acrylamide (Oster, 1954; Oster, Oster and Prati, 1957) as a potential embedding material for sectioning tissues. Ornstein who had been using starch gels for electrophoresis realized that polyacrylamide had better properties than starch for electrophoresis. It was tried out in Ornstein's laboratory by B.J. Davis and results looked so promising that they were reported by Ornstein in a published discussion following a paper delivered in 1959 (Ornstein and Davis, 1959).

Quite independently of this report, Raymond and Weintraub (1959) suggested the use of acrylamide as a supporting medium for electrophoresis. A further paper by Raymond and Wang (1960), soon afterwards gave further details of the method.

Since its introduction polyacrylamide gel has become increasingly popular as a medium for electrophoresis, and has been used under several conditions for many different purposes, some of which are described in Table I.

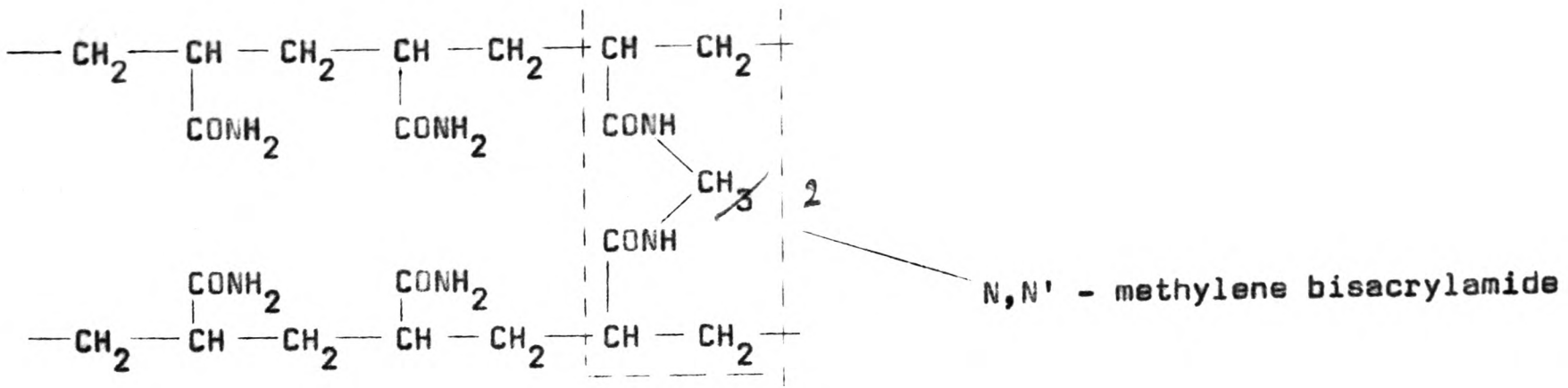
The method has extensive medical applications, most of which involve a comparison of proteins from normal and diseased tissues.

In 1964 a conference was held to discuss the lines of research involving the use of gel electrophoresis. The papers given at this conference were published in the Annals of the New York Academy of Sciences, Volume 121, Article 2.

Acrylamide ($CH_2{:}\overset{CH.}{C}CONH_2$) or propenamide is a water soluble vinyl monomer of molecular weight 71.08 and melting point 84^{o}. With a derivative of acrylamide, N,N'-methylene bisacrylamide as a cross-linking agent, it can be polymerized into gels which are linear polymers consisting of long hydrocarbon chains in three dimensions.

Table I. Some of the purposes for which electrophoresis on polyacrylamide gel has been used.

Study	Name	Year	Gel Conc.	Further details
Serum proteins	Raymond & Weintraub	1959	5%	Tris buffer at a range of concentrations
Basic proteins & peptides	Reisfeld Lewis and Williams	1962	15% + 7.5%	Balanine/acetic acid
Histones	Cruft	1962	10%	Acetate buffer: protein pattern resembled that on moving boundary electrophoresis rather than starch gels
Fungal proteins	Chang Srb & Steward	1962	7.5%	tris/HCl, tris/glycine
Plant proteins	Racusen & Calvanico	1964	5%	NH_3 - NH_4Cl
	Steward & Barber	1964	7.5%	tris/HCl, tris/glycine
Enzymes	Appel et. al.	1965	5%	β-galactosidase in E. coli proteins
	Allen & Gockerman	1964	10%	acid phosphatate of liver tissue
RNA	Richards & Gratzer	1964	10%	tris/barbital buffer. Migration rate is correlated with sedimentation coefficient.
	Loening		2.4%	tris/sodium acetate/EDTA Separated 4S, 18S, 28S RNA and DNA
Genetic Studies	Fox, Thurman & Boulter	1963		Legumes
	Kates & Goldstein	1964		Amoeba
	Johnson & Hall	1965		Triticinae



The cross links are considered not to affect the pore size (Ornstein, 1961) but serve only to stabilize the mass of intertwined chains against convection.

The amide groups at intervals make it hydrophylic. There may be some hydroxyl groups or acidic groups at the ends of the chains, but there are no phenolic, carboxylic, sulphydryl or amino groups. Because they are non-ionic there is no backward endosmotic flow, so markers are not needed. To find the separation ratios, the relative rates of migration can be used. The rate of diffusion in acrylamide gels is extremely slow.

The pore size of acrylamide gel can be varied by varying the concentration of acrylamide, the effective pore sizes used ranging from 400Å to 10Å. The relationship between concentration and pore size has been stated by Ornstein (1964).

Polyacrylamide gels are transparent, strong and chemically inert. The latter property leads to difficulties when it is necessary to dissolve them. The gels can be dehydrated and rehydrated. Thin sheets of polyacrylamide can be dried onto glass after they have been soaked in 2% glycerol. This makes storage and reference easy.

It is not possible to recover protein from acrylamide gels by freezing and thawing, which is the method employed for starch gels, because polyacrylamide gels are stable to freezing. To recover proteins intact from gels for further investigation, e.g. for re-electrophoresis, elaborate procedures have to be used, involving elution from fragments by diffusion,

or from gel slices by embedding them in dilute gel and removing the proteins by electrophoresis. Continuous elution procedures have been evolved but these have the disadvantage that slow moving proteins are collected in large volumes of eluting buffer so are considerably diluted (Racusen and Calvanico, 1964).

The difference between gel electrophoresis, and electrophoresis on any other media is that gels exert a frictional resistance to the particles moving through, i.e. there is a sieving effect on the particles. This effect was considered for liquids by Synge and Tiselius, (1950), and established for gels by Smithies, (1955). The mobility of a particle moving through a gel depends not only on the net charge of the particle but also on the size of the particle. The higher the net charge and the smaller the particle the faster it will travel through the gel. The reduction in the rate of migration when the concentration is increased, is not simply related to free volume because the effect is different with different proteins (Raymond and Nakamichi, 1964). The relative rates of migration of serum proteins have been predicted by Ornstein from a theoretical consideration of the mobility of the molecules in water, the molecular weight and the diameter.

Acrylamide gels may be cast in sheets for electrophoresis horizontally or vertically, or can be cast in small tubes for vertical electrophoresis. The latter method is called DISC electrophoresis because this technique depends on <u>disc</u>ontinuities in the electrophoretic matrix (two concentrations of gel are used, with buffers of two or more pH values) and coincidentally because of the <u>disc</u>oid shape of the separated zones of ions.

Disc electrophoresis has the advantage that dilute protein samples can be used because there is an automatic concentration of the protein into thin starting zones when electrophoresis is commenced. This results in high resolution of the components even after short runs.

Details of the method of Disc electrophoresis were described by Ornstein (1961) in a preprint made available by Distillation Products Industries Division, Eastman Kodak Co., Rochester, N.Y. Modifications of the method for separating very large quantities of protein have been made by Ornstein, O'Brien and Davis (in preparation). For separating extremely small quantities of protein (picogram amounts) acrylamide gel fibres 10 microns wide, immersed in non-polar medium can be used (Ornstein, in preparation).

The arrangement of gels in the tube for disc electrophoresis recommended by Ornstein is shown in Figure I. Under the influence of an electric field proteins move downwards out of the sample gel in which they are applied, into the spacer gel, through this and through the fine pore gel where they separate into ionic groups according to their mobilities. They move more slowly in the fine pore gel where the bands of protein separate from each other.

The theory of the method, put forward by Ornstein depends partly on the use of a discontinuous system of buffers. The mobilities of the ions of the buffer are arranged so that the protein sample occupies a region between a buffer containing rapidly moving ions below it, and a buffer containing slowly moving ions above it. When electrophoresis is started, the proteins will be concentrated into very fine zones arranged in order of their mobilities and stacked up between the two buffers. As they pass into the running gel, the conditions are arranged so that the ions in the buffer above the proteins increase in mobility. At the same time the proteins are slowed down because they pass into a gel which has smaller pores, and therefore offers much higher frictional resistance. The net result of this is that the buffer front overtakes the protein zones and catches up with the buffer below the protein.

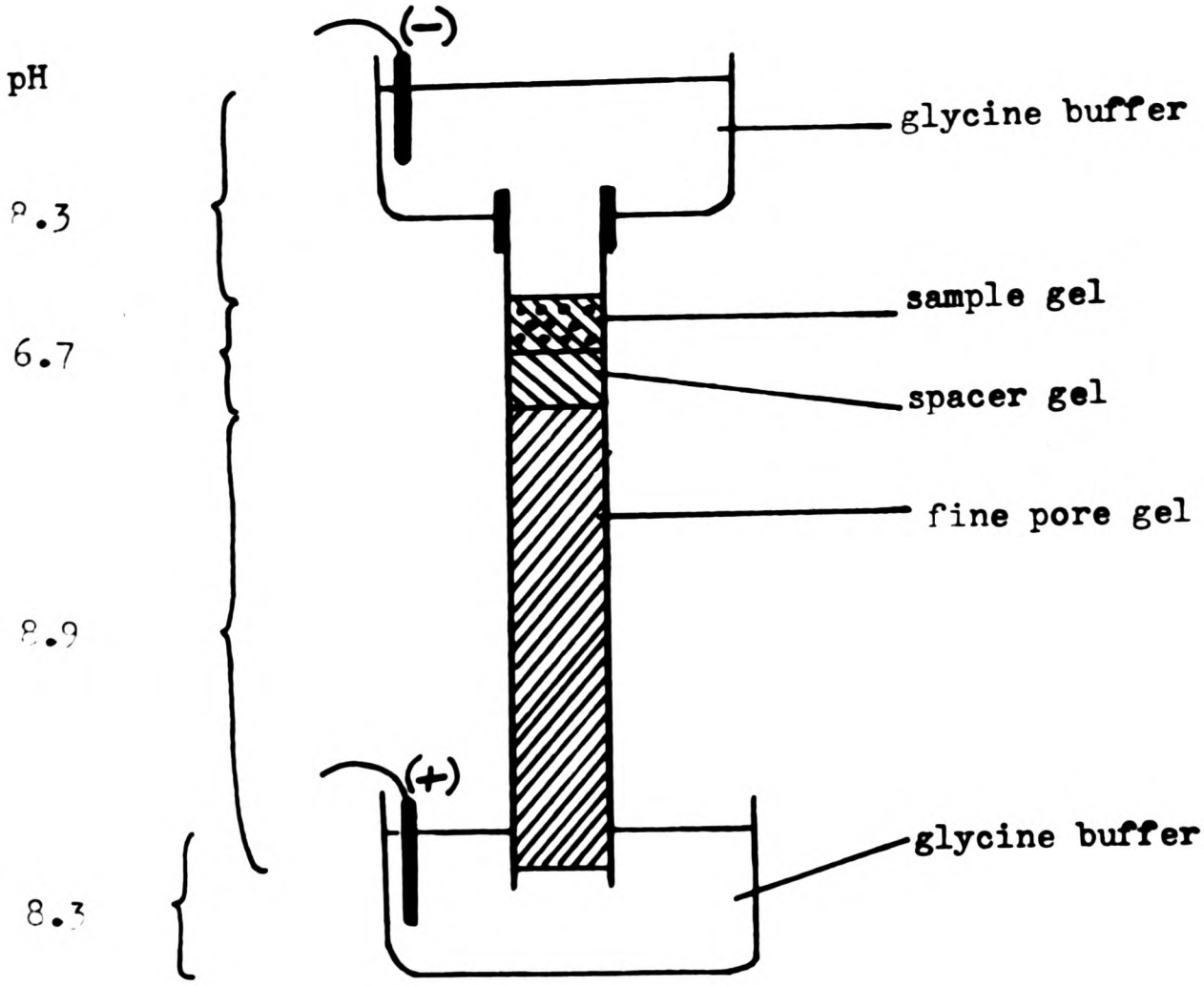


Figure 1. The arrangement of polyacrylamide gels for disc electrophoresis (Ornstein, 1961).

The protein zones, no longer held together by the pull of the electrostatic field, separate and progress through the running gel at different rates. This results in a series of sharply defined protein zones which show up, on staining, as bands.

Poulick (1957), using a discontinuous buffer system with starch gels, suggested that protein bands were sharpened when overtaken by the buffer front, at which there is a drop in potential. A comparison by him, of this system with a continuous buffer system, showed that in the latter case there was no drop in potential along the gel, nor was there good resolution of the protein components.

However, on acrylamide, bands as sharp as those achieved by Ornstein can be obtained with a continuous buffer system, i.e. the same buffer in the electrode vessel, sample, spacer and fine pore gel (Raymond, 1964). Furthermore, the sample gel and spacer gels which are, according to Ornstein necessary for a good separation of proteins, can be omitted with apparently no loss of resolution. A combination of continuous buffer system with the use of just a fine pore gel (Raymond, 1964) will also produce good results, and it is this system (with the addition of a spacer gel) which was used in the present study.

(vii) Enzyme tests.

Having obtained a general impression of the protein changes which occur during differentiation, it was then instructive to examine one or two proteins in particular to find out how they changed during differentiation. For this investigation, some enzyme proteins were chosen. Although a study of enzyme proteins was earlier criticized on the basis that any particular enzyme represented only a minute fraction of the proteins present, it seemed legitimate at this stage to examine enzyme

activities so that the identity of some components of the protein pattern might be established.

Methods have been established for testing gels for the activity of several enzymes including alkaline phosphatase, (Levinthal, Signer and Fetherolf, 1962), acid phosphatase (Allen and Gockerman, 1964) and dehydrogenases (Sundaram and Fincham, 1964; D.A. Thurman, personal communication).

It should be possible, by testing for a large number of enzymes, to identify some of the protein fractions with certain enzymes. But the number of enzymes in a cell is so great that it would be necessary to do a large number of enzyme tests to show that any one band did not contain more than one enzyme. To make a thorough investigation of the identities of the protein bands it would be necessary to cut them out, and re-run them in different conditions of pH or gel concentration and then to stain for total protein and enzymes.

A phosphatase was chosen as a representative of carbohydrate metabolism but, as with most esterases, the phosphatases have such a broad substrate specificity that they probably consist of families of enzymes. Some dehydrogenases were chosen because they are substrate specific, and therefore more likely to be specialized enzymes rather than families of enzymes.

(viii) Growth in culture

When the protein changes which take place during differentiation in the intact root had been studied, the changes which take place in root segments during culture were investigated. The system of excision and culture is equivalent to a step-down system in bacterial culture, because the root segments, which previously had been receiving nutrients

and growth substances from the rest of the seedling, are suddenly deprived of these and have only the supplied sucrose in the medium. It is therefore a situation of interest in which the growth of regions of the root can be studied after the influence of the rest of the seedling has been removed.

(ix) Incorporation of precursors into protein.

The protein bands fractionated on a gel represent the net amount of the various soluble proteins present in the cell at the time it was examined. The relative rates at which the proteins are being synthesised may differ considerably, the protein visible on the gel representing the difference between the amount of protein synthesised and the amount broken down. To examine the relative rates of synthesis of the protein bands, it was necessary to follow the incorporation of a labelled protein precursor into the soluble proteins. Since the soluble proteins have been produced by a protein-synthesising mechanism in one or other of the particulate cell fractions, as a preliminary to this, the rates of incorporation into cell particles of intact and excised root segments was studied, to find out which particulate components were responsible for this synthesis. Experiments consisted of incubating the roots of the intact pea seedlings in a medium containing a ^{14}C-labelled amino acid (leucine) and after subsequent homogenisation and fractionation, the protein was purified, and the radioactivity measured.

(x) The effect of an antibiotic on protein synthesis.

The incorporation of leucine into the proteins of root segments which had been pretreated with an inhibitor of RNA synthesis (actinomycin D) was also studied to find out if protein synthesis in the pea root depends on a continuous synthesis of RNA.

II. METHODS

(i) Electrophoresis on polyacrylamide gel.

(a) Development of the method.

When this work was started, there were few papers available on the subject of polycrylamide gel electrophoresis and none gave complete details of the method, because Ornstein who had introduced the method was going to publish all the details. The paper by Ornstein was not published until 1964, and the preprint of this paper describing techinical procedure, which was being made available by Canalco Industries Division, I did not discover until several months after the work was commenced. Consequently initial experiments were very much trial and error.

Acrylamide can be polymerized either by a chemical method or by an 'optical' method. The latter involves exposing a solution of the acrylamide monomers, with catalytic quantities of riboflavin, to light. In the chemical method, the addition of two catalysts to the monomer solution results in rapid polymerization. The catalysts used are: ammonium persulphate, and either N,N,N',N'-tetramethylethylenediamine (TEMED) or dimethylaminopropionitrile (DMAPN).

First experiments were designed to find which of these methods provided the most suitable gel for separating pea root proteins. The chemical method brought about polymerization very quickly (in about 5 minutes) while the optical method (recommended by Steward & Barber, 1964) brought about polymerization in about 45 - 60 minutes.

When the gels were used for electrophoresis, neither produced satisfactory results. In an attampt to achieve a successful separation of proteins, several factors were varied. These included the concentrations of the monomers, the amount of catalysts, the intensity of the light

(in the optical method) the buffers used and their concentrations - all without success. It appeared that the gels were not properly polymerized. Occasionally air bubbles formed in the gels and around these, areas of the gel remained unpolymerized. It was then realized that air was inhibiting the polymerization so the solutions were boiled under reduced pressure to remove air before use. The method immediately improved, so all alterations in the method which had previously been made, were abandoned and the original methods used.

The chemical and optical methods were again compared and the chemical method chosen, because, in contrast to Steward's findings better results were obtained on the chemically polymerized gels. The reason for this difference in findings may lie in the acrylamide monomer. Kodak acrylamide when recrystallised produces different results from the non-recrystallized material (Loening, p.c.) and it is possible that acrylamide obtained from different manufacturers may differ in its purity.

The method which was adopted after several preliminary attempts is described below. This is the method which was used routinely. Any deviations from this method will be stated when those experiments are described.

The gels were prepared in glass tubes of 0.6 cms internal diameter and 7 cms long. Two concentrations of gel were used: the spacer gel (2.5%) in which the proteins sort themselves out according to their mobilities and become concentrated into discs and a fine pore gel (7.5% having an average pore size of 50 Å), in which the discs are separated.

The gels were made up in a buffered solution and catalysed chemically. The spacer gel:

2 ml acrylamide solution containing 5% acrylamide, 1% N,N'-methylene bisacrylamide.

2 ml buffer 0.1M tris/0.1M glycine pH 8.9

0.06 ml 10% TEMED in ethanol

0.06 ml 10% ammonium persulphate in water

The fine pore gel:

5 ml acrylamide solution containing 15% acrylamide and 0.3% N,N'-methylene bisacrylamide

5 ml buffer 0.1M tris/0.1M glycine pH 8.9

0.1 ml 10% TEMED in ethanol

0.1 ml 10% w/v ammonium persulphate in water

The buffer used in the electrode vessels was 0.05M tris/0.05M glycine pH 8.9. The tracker dye was a 0.02% solution of bromophenol blue in buffered sucrose medium.

The staining solution contained 0.05% Amido black dye in methanol/acetic acid/water in the proportion 5:1:5 and the destaining solution was methanol/acetic acid/water in the same proportions but without the dye. After a prolonged washing in the destaining solution the gels were stored in 7% acetic acid in water.

(b) Procedure.

The glass tubes which had scratch marks on them at 4.5 and 5.1 cms from the base, were stoppered with rubber bungs (size 6) and placed in a rack which held them vertical. The fine pore gel was prepared first. The acrylamide solution and buffer were mixed and oxygen removed by boiling under reduced pressure. The catalysts were added and the solution mixed by drawing it in and out of Pasteur pipette carefully, avoiding introducing air bubbles into the solution. The tubes were rinsed out with acrylamide solution to wet the corks and prevent air bubbles forming when the tubes were filled. The solution was pipetted into the tubes until they were filled to the first mark. Then, very carefully, using a finely pointed

Pasteur pipette, some degassed buffer (0.05M tris/0.05M glycine) was layered onto the surface of the acrylamide solution. If any mixing occurred, the gel was discarded, because the gel surface would have been unsatisfactory.

The layer of buffer removed the meniscus of the acrylamide so that the gel when set had a flat surface. It also excluded air from the solution which would otherwise have prevented polymerization of the surface layers. The whole operation, after the addition of catalysts had to be carried out quickly because the gels set in a few minutes. During this time the boundary between the acrylamide solution and the buffer disappeared and a new one formed a few millimeters lower, at the gel surface, when polymerization took place. This was due to oxygen inhibition of polymerization in the surface layers. The slower the method of polymerization used, the lower did the gel surface form.

The spacer gel was prepared in the same way. The monomer solutions and buffer were mixed, and boiled under reduced pressure to remove the air. At this stage the buffer was removed from the surface of the gels with strips of filter paper. (If a layer of buffer was left on the surface of the fine pore gels, the spacer gel would not stick to it.) The catalysts were added to the buffered acrylamide solution, mixed, and this pipetted into the tubes until they were filled to the second mark 5.1 cms from the base. Buffer was layered onto the surface of the acrylamide solution as before.

When the spacer gel polymerized it was not transparent like the fine pore gel, but was cloudy due to the relatively high concentration of the cross linking agent (N,N'-methylene bisacrylamide). The gels were allowed to stand for 1-2 hours before use. The buffer was shaken off the surface of the spacer gel, and the rubber bungs removed. The latter

operation had to be done carefully without causing a vacuum which would pull the gel away from the walls of the tube. The side of the bung was depressed with the thumbnail and air allowed into the small space formed under the gel. Then with the bung still depressed it was removed slowly. The bottom ends of the tubes were filled with the buffer which was to be used in the electrode vessels. In this case it was 0.05M tris/0.05M glycine. The ends were filled until a hanging drop appeared.

The tubes were fitted into grommets of the upper electrode vessel which was held on a ring clamped at the top of a retort stand to make the operation easier.

The protein sample which consisted of about 200 ug of protein in 0.1 ml was pipetted onto the surface of the spacer gel. The protein solution which was usually used consisted of the soluble proteins of root cells in a sucrose medium. If the protein was not in a sucrose solution, a few crystals of sucrose were added to the protein samples before application, to increase their density, and facilitate overlayering of buffer.

Several workers (Ornstein, 1961; Chang et.al., 1962; Reisfeld et. al., 1962) recommended polymerizing the protein sample into a gel, but I found that the presence of the protein inhibited the polymerization of the gel. The point of doing this was to avoid convection currents in the protein sample when the current was supplied, but no convection currents were seen in the protein applied in sucrose.

When a protein had been applied, a drop of bromophenol blue in buffered sucrose solution was added. This was to act as a tracker, moving down the tube just ahead of the fastest proteins. The progress of the electrophoresis could thus be followed.

The tubes were filled with buffer using a finely pointed Pasteur pipette to avoid disturbing the protein solution. The electrode vessels were then filled to depth of 4 cms with buffer.

The upper vessel was lowered until the bottom ends of the tubes were submerged by about 1.0 cm. The terminals were connected so that the lower one was made the anode, and a current of 4 mamps per gel column supplied. Steward and Barber (1964) found it necessary to apply 2 mamps per column until the dye had reached the spacer (in cases where the sample gel failed to polymerize), to prevent loss of protein into the upper buffer compartment due to convection in the liquid sample gel. In my system, the dye sharpended to a fine line suggesting that there was no convection, while still in the sucrose (sample) solution when 4 mamps were supplied at the commencement.

With 4 mamps/gel column the initial voltage was 180 V but rose to 250 V during the electrophoresis. With constant voltage the current fell off during the run, so the time taken was longer. All electrophoreses were carried out at constant current.

Electrophoresis was continued until the tracker dye had reached the bottom of the tube. This took 25 - 30 minutes, after which time the temperature of the gel had risen to 37^{o}C. With the tris/glycine buffer system, the band of tracker dye remained sharp for the first 10 minutes or so, then became rather diffuse, but its progress could still be followed.

If the dye travelled faster in some tubes than in others, these were corked when the dye reached the bottom, and the current adjusted to continue supplying 4 mamps per tube to the rest. When the dye reached the bottom in all the tubes the electrophoresis was stopped.

The gels were removed from the tubes by rimming between the gel and the glass with a fine strong wire. The buffer which remained in the tube

after they had been removed from the grommets served to lubricate the gels as they were being extracted.

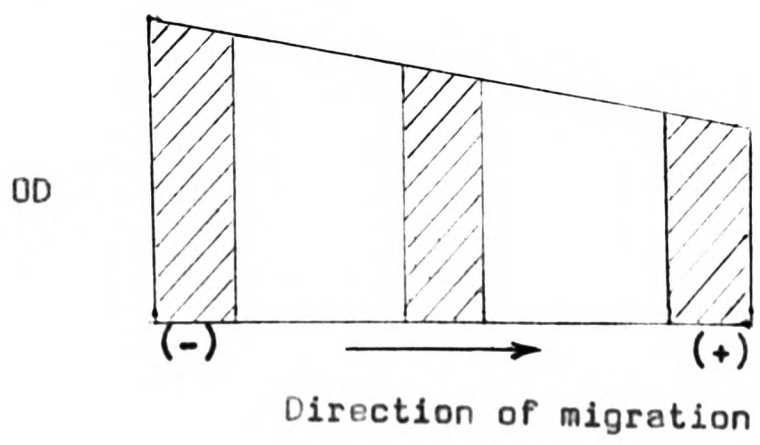
(ii) Staining gels.

The gels were stained in methanol/acetic acid/water containing amido black for 1 - 2 hours. Excess stain was removed from the gels by washing them for 1 - 2 days in the methanol/acetic acid/water without dye.

When excess dye had been washed out of the gels the protein components appeared as dark blue bands which showed up against a background staining. The protein bands on one gel formed a pattern which was characteristic for a given protein mixture.

The intensities of the bands depend on the amount of dye taken up by the protein. This amount will depend on the stain used whether it is an acidic or basic dye, and on the number of reactive sites in the protein. The dye used, Amido black 10B, is an acid dye, and will bind to the amino groups of the proteins. Since the mobility of a protein depends partly on the ionization of these same groups, a protein which takes up more stain will also be expected to move more slowly (in the conditions of pH used here). This means that there will be a tendency for bands at the top of the gel to take up more stain (for the same amount of protein) than bands at the bottom.

Bands containing equal quantities of protein will be expected to stain as show in Figure 2.



The general intensity of staining does follow this pattern, but there are feint bands at the top of the gel, and fast running bands which stain intensely.

Since the mobility of proteins also depends on the relative number of COOH groups, and on the size of the protein it is likely that any bias towards the staining of slow-moving proteins will be slight.

It was found that on several gels some vertical streaks appeared though they were not very prominent. They appeared on the outside of the gel and were presumably due to protein leaking down between the gel and the wall of the tube, although gels seem to adhere closely to the glass. The streaking did not spoil the scans.

(iii) Scanning gels.

Gels were scanned with the chromoscan densitometer (Joyce, Loebl and Co. Ltd.). The principle of this instrument is as follows: a gel is moved in front of a narrow beam of light so that the light beam, a narrow slit, passes along the long axis of the gel. The travel of the sample is synchronized with the movement of the paper in the pen recorder.

The light beam is chopped by a rotating mirror, into two beams. One of these passes through the sample onto one photocell, the other passes through an optical wedge onto a second photocell. The optical wedge moves until the light falling on both photocells is balanced, converting the transmission to an optical density value. A pen is mechanically connected to the optical wedge so the further the wedge moves the further the pen is moved.

The gels were scanned in a parallel-sided holder which reduced the curvature effect of the cylindrical gels.

The range of the optical wedge used was equivalent to 0.5 - 2.0 optical density units. For scanning smaller changes in optical density a more sensitive wedge was available.

Filters chosen were complementary to the colour to be scanned. This meant that the light transmitted by the filter was absorbed by the specimen. If a filter is exactly complementary all the light transmitted by it will be absorbed by the gel. Most filters transmit over a wider range of wavelengths than the light absorbed by the dye, so there will be some leakage of light.

It was important to know if the height of the peak drawn by the pen was proportional to the density of colour in that peak. It was expected that there would be a range over which the response would be linear, and in this range a doubling of the intensity of the dye would produce a peak of twice the amplitude.

A range of concentrations of amido swartz dye were made up and their optical density measured on the Unicam Spectrophotometer. The same solutions were then put into the Chromoscan densitometer and the density (i.e. movement of the pen) recorded on the graph paper. The experiment was carried out at two sensitivities, with and without coloured filters. The reason for doing the test also without a colour filter was that it had been found that reasonable scans of gels which had been overloaded with protein could be obtained if the colour filter was omitted.

The height of each reading on the Chromoscan densitometer above the base line was measured by counting the squares and the values were plotted against the concentration of dye and against the reading on the Unicam (Fig. 3).

The results were almost straight line graphs. This was surprisingly good especially for the values obtained without a colour filter, and means that the Chromoscan densitometer works much better in

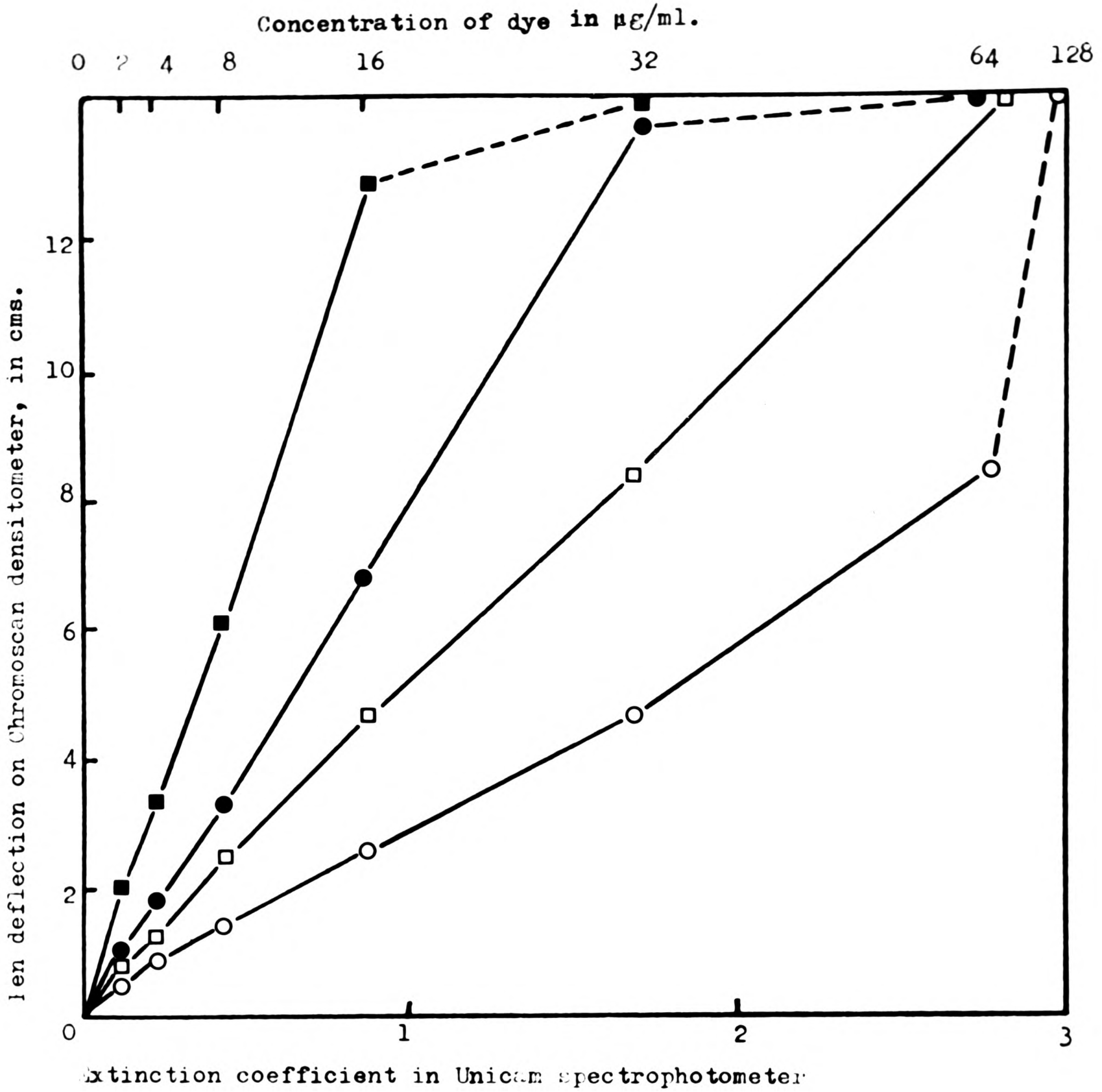


Figure 3. The relationship between the pen deflection on the Chromoscan densitometer and the extinction coefficient of Amido Black dye in methanol/acetic acid/ water, 5:1:5, measured in a 1 cm cuvette.

——■—— Sensitivity cam 3 + colour filter with peak transmission at 600 mµ.

——●—— Sensitivity cam 1 + colour filter.

——□—— Sensitivity cam 3 with no colour filter.

——○—— Sensitivity cam 1 with no colour filter.

practice than it should in theory.

It had previously been thought that removing the colour filter would decrease the proportionality of the response, but since the height of the peaks is proportional to the density of the dye, in this case too it is more or less proportional to the concentration of protein.

Because the response of the Chromoscan densitometer is so proportional, gels with slightly different amounts of protein could be compared.

Figure 4 shows the scans obtained when gels with different concentrations of protein were scanned at different sensitivities. The differences between each pair of scans illustrated the variation between gels. Some of this may be attributed to the fact that there are different concentrations of protein on the gels but similar variation is often obtained from different gels on which the same amount of protein has been run.

(iv) Photography.

Photography of the gels has proved very difficult because of the curvature effects of the glass tubes in which they are contained, and the fact that light reflects off the glass. However, photographing the gels out of the tubes (floating them in 7% acetic acid) produced very blurred pictures. It was necessary to photograph them in the tubes because these had the effect of magnifying the gels in the lateral dimension and made the bands more easily visible.

To reduce the curvature effect and reflection from the tubes they were photographed from a distance of about 3 feet using an Edixa,

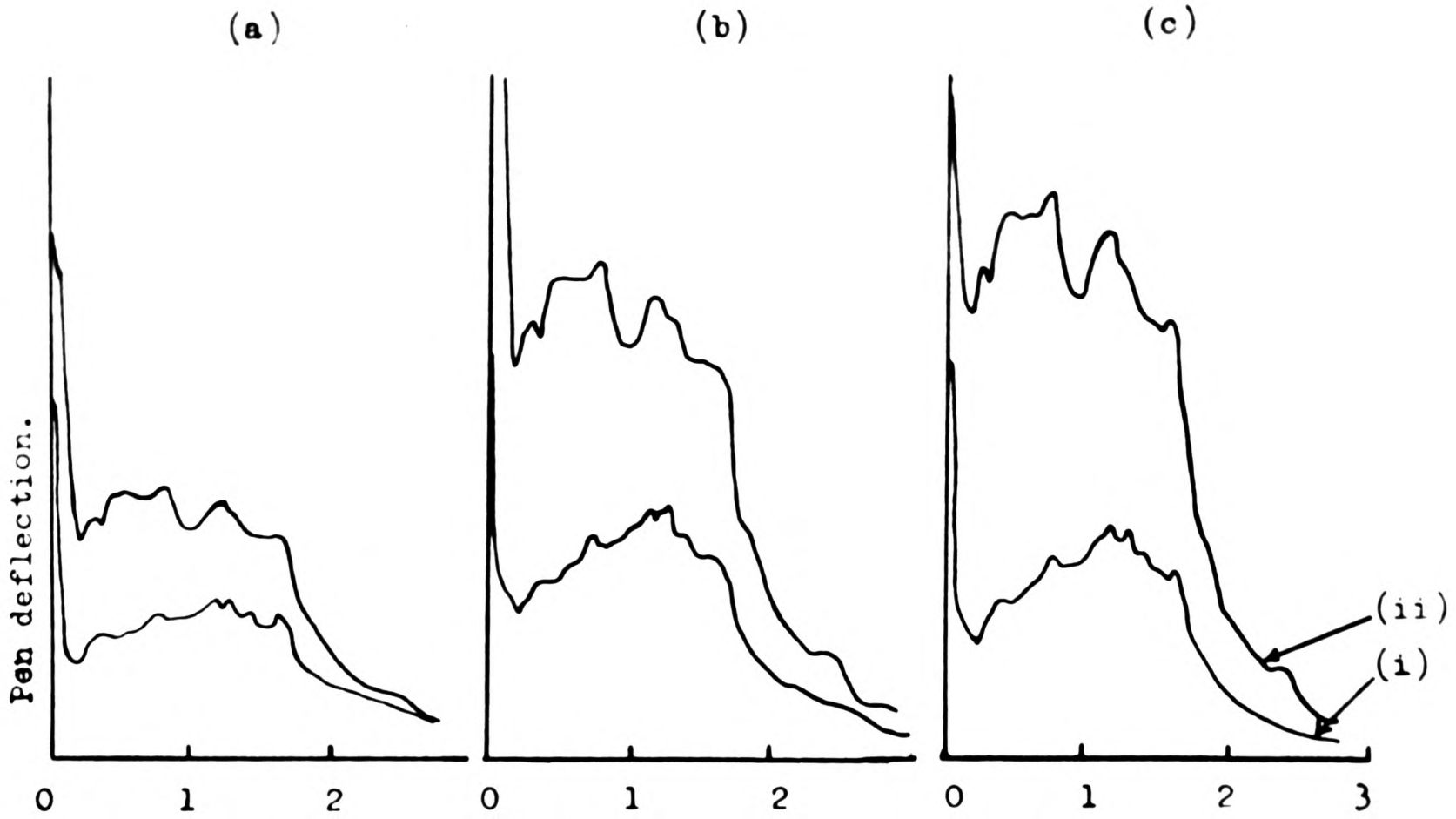


Figure 4. Gels on which (i) 100 µg and (ii) 200 µg of protein have been fractionated. Scans were made at different sensitivities: (a) With sensitivity cam 1 and no colour filters, (b) with sensitivity cam 3 and no colour filters, (c) with sensitivity cam 1 + colour filters (maximum transmission 600 mµ).

35 mm. camera fitted with a Zeiss long focus lens (f = 16.5 cms.) against an illuminated background of diffused light. Reflection was minimised by having a screen round the gels to cut out light arriving at the tubes from various angles. They were photographed through a tricolour red filter using a micro neg pan film which is a very fine grain high contrast film.

The density range of the dye in the gel was such that successful photographs could only be made using a mask (an out of focus positive) in conjunction with the negative.

(v) Preparation of the plant material.

(a) Planting method.

The peas used in all experiments were Pisum sativum var Meteor obtained from Suttons and Sons, Reading, and later from Dobbies, Edinburgh.

The peas were sorted, and any cracked or infected ones removed. The good ones were soaked in running tap water for about 16 hours. After this time most of the peas had imbibed and swollen. Any which had failed to imbibe were discarded.

The imbibed seeds were planted in damp vermiculite (2 parts vermiculite, 1 part water) and grown in the dark at 22.5^{o}C for 48 hours, after which time the roots were about 3.5 cms in length.

(b) Harvesting pea seedlings.

The pea seedlings were harvested carefully, and damage to the roots avoided. If they were to be used for a protein preparation they were washed in tap water and placed in ice cold water. If they were to be

incubated either in culturing experiments or for pulse and chase experiments they were washed with water at 25°C and subsequent procedures carried out at 25°C.

(c) Root cutting.

Pea roots were sectioned either at room temperature or at 0°C - 4°C depending on the experiment. In some cases the 7 mm root tips were excised, in other, the roots were cut into the following three segments:

Segment 1, 0 - 1.6 mm. This is the apical segment consisting of the meristematic cells.

Segment 2, 1.6 - 3.4 mm from the apex. This segment includes the expanding cells.

Segment 3, 3.4 - 6.4 mm from the apex. This segment consists of cells which are nearly mature.

These segments are same lengths as those used and described by Loening (1961).

The roots were sectioned using a device which consists of several perspex sheets of appropriate thickness with holes drilled through them to hold the roots. The sheets clamped together, were laid flat on a piece of glass and roots inserted into the holes until they touched the glass. The bases of the roots which protruded from the surface were sliced off level with it and the sheets, still clamped together, turned over. The first sheet which was 1.6 mm in thickness was removed and the root apices were left protruding from the surface. These were sliced off level with the surface, and transferred to an ice cold tube. The next sheet, 1.8 mm in thickness was removed and so on.

For smaller segments (e.g. of 1 mm) a microtome was used.

(vii) Homogenization and centrifugation.

The cut segments were placed in cold thick walled glass centrifuge tubes and all subsequent procedures carried out at 0 - 4°C. 1 ml of buffered sucrose medium was added to each tube containing twenty 7 mm root tips, or fifty of the smaller segments. When 1 mm segments were cut, 50 were used per 0.75 ml of medium.

Homogenization media of two compositions were used. The first contained 0.5 M sucrose, 30 mM tris, 24 mMHCl, and 5 mM $MgCl_2$. The second had the same molarities of sucrose, tris and HCl, with 1 mM $MgAc_2$ and 50 mM KCl.

The root tips were homogenized quickly with a loose teflon pestle rotating at up to 1,000 rev./min., for just long enough to break most of the cells. The homogenate was centrifuged as follows:

(1) 1,000 x g for 5 minutes. This precipitated the debris which included some unbroken cells, cell walls and nuclei (90% of the total cell DNA is found in this fraction).

(2) The supernatant from (1) was centrifuged at 12,500 x g for 15 minutes. The precipitate was the mitochondrial fraction and electron micrographs show it to consist of at least 60% mitochondria.

(3) The supernatant from (2) was centrifuged at 30,000 x g for 30 minutes and the precipitate called fraction X (Leoning, 1961). This pellet consists of membranous material and probably polysomes (G. Bull, unpublished data).

(4) The supernatant from (3) was centrifuged at 130,000 x g for 90 minutes. The supernatant from thisspin consists of the soluble proteins of the cell. It was stored at -20°C until needed.

The pellet from this spin (4) consists of 2 components both of microsomal origin. These were separated into a ribosomal pellet and a

vesicular microsome pellet as follows:

After decanting the supernatant, the tube containing the microsomes was dried carefully with filter paper (a hard grade (50) which left no fluff on the tube). It was then inverted for 15 minutes after which time the two components could be seen. One was small and transparent in the position of the original pellet, and the other larger and more cloudy, beginning to slide down the tube. The small pellet consisted of free ribosomes (R) and the cloudy pellet of vesicular or membranous microsomes (V). This method of separating the microsome pellet into two fractions was first used by Loening (1961).

To separate the two pellets completely, the tube was placed, still inverted, into a conical centrifuge tube and this centrifuged at 200 - 500 rev./min. for a few minutes. Fraction V was thus transferred to the base of the conical tube. Occasionally, as the soft pellet slid down the tube it left a smear of material on the wall of the tube. For quantative measurements this was removed by rubbing it with a drop of sucrose medium on a fine glass rod, and recentrifuging the tube.

(vii) Electron microscopy technique.

Osmium staining.

The pellets obtained after differential centrifugation were fixed and embedded as follows:

They were fixed for 2 hr in 5 ml homogenizing medium (conc x 2)
1 ml water
4 ml 5% osmium tetroxide in water

15 mins	25% meths at 0°C
15 mins	25% meths
30 mins	50% meths
30 mins	75% meths
30 mins	100% alcohol brought up to room temp.
30 mins	100% alcohol
15 mins	50/50 alcohol/propylene oxide
15 mins	propylene oxide
15 mins	propylene oxide
2¼ hours	25/75 propylene oxide/complete araldite mixture
15 hours	araldite at 68°C

The araldite mixture was: araldite CY 212 .... 27 ml

tetrapropenyl succinic anhydride .... 23 ml

(dodecenyl succinic anhydride)

DMP-30 .... 0.9 ml

Sections were cut with an LKB microtome, lead stained, and photographed, (Fig. 5).

During the osmium fixation the vesicular pellet of the microsome fraction floated off, and the ribosomes resuspended. Another attempt was made to fix the microsome pellet, in position so that sections could be cut through V and R. It was found that a drop of agar (made up in homogenizing medium) would hold the pellet in position.

Gluteraldehyde/osmium fixation. The gluteraldehyde reacts with tris (present in the homogenizing medium and therefore in the pellets) so they were resuspended and centrifuged in potassium phosphate medium containing $MgAc_2$(1mM), KCl(50mM), Sucrose (0.5M), K_2HPO_4(0.05M), and KH_2PO_4(0.05M).pH7.5

The pellets were fixed overnight in gluteraldehyde (1%) in phosphate buffered medium as above followed by 3 washes in phosphate medium 1 hour each and stained in osmium (1.25%) in phosphate buffer.

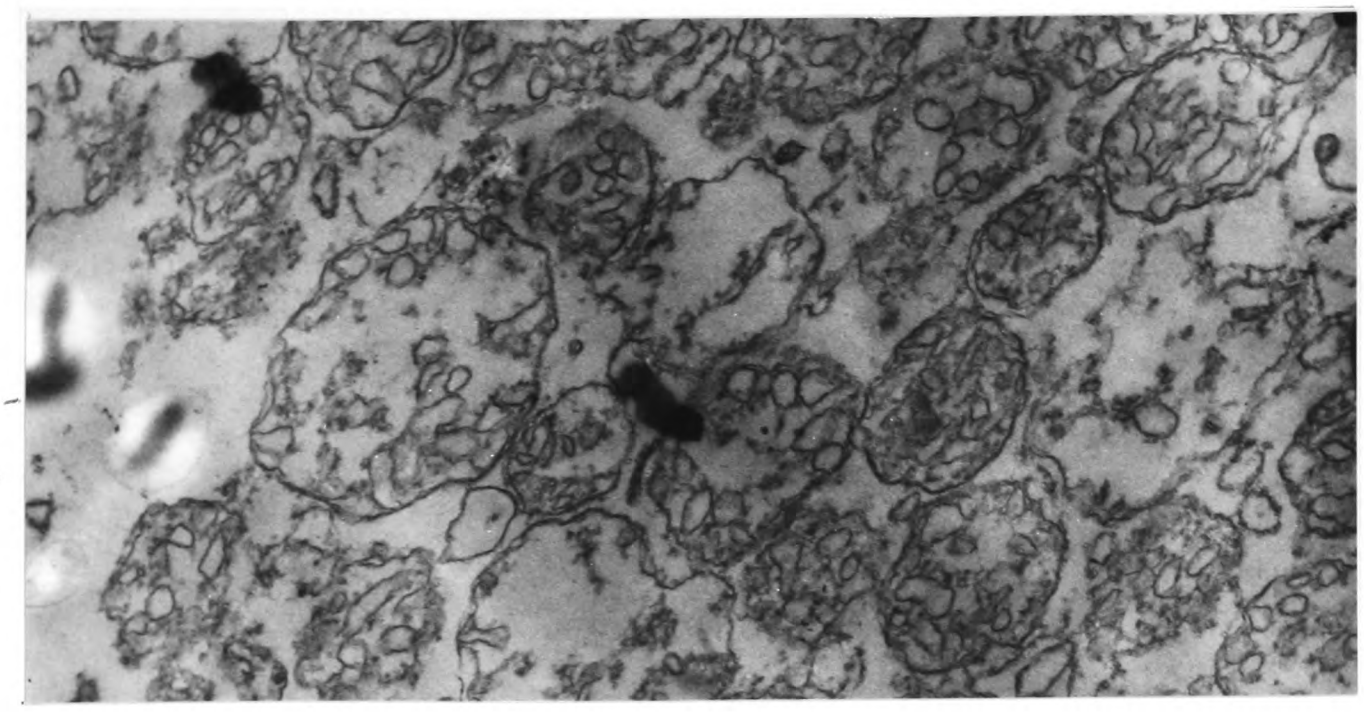
Figure 5. Electron micrographs of the particulate cell fractions. The sections shown were cut from pellets which had been fixed with osmium tetroxide and stained with lead.

Figure 5 (a) shows the components of the mitochondrial fraction. These include some membranous material besides mitochondria.

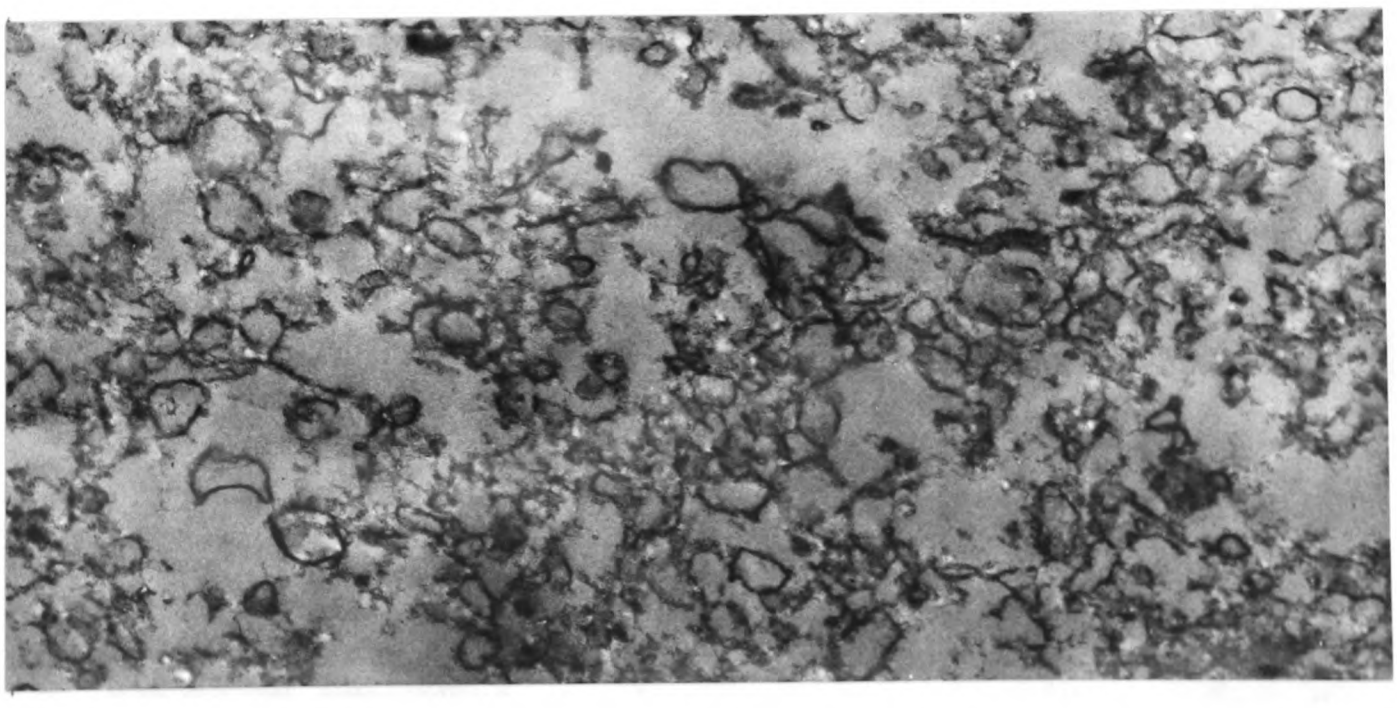
Figure 5 (b) is of fraction X. This consists of membranous material and some clusters of ribosomes which could be seen, at higher magnifications, associated with the membranes.

Figure 5 (c). Fraction V. At the outside of this pellet, close associations of membranous structures were seen. Further inside, the pellet was less heterogeneous (Figure 5 (d)) and consisted of clusters of ribosomes and small vesicles.

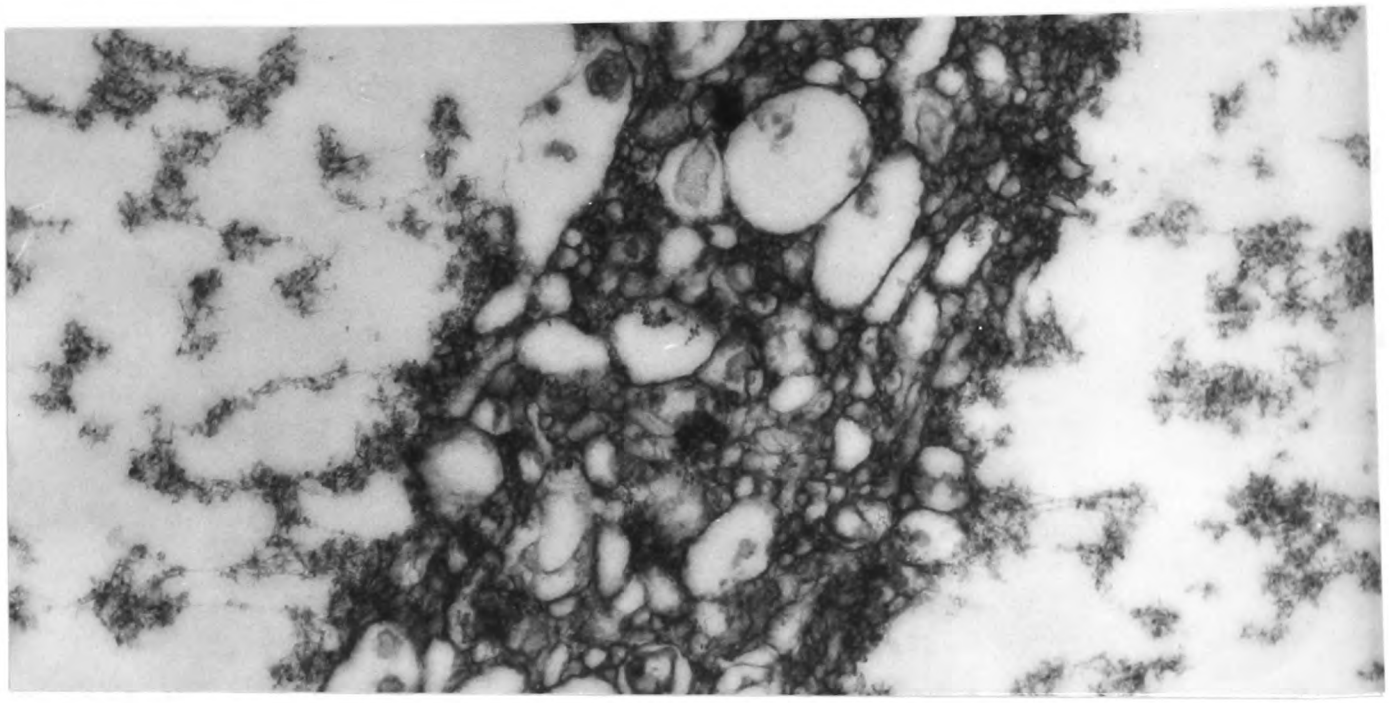
The ribosome fraction consisted of free ribosomes.



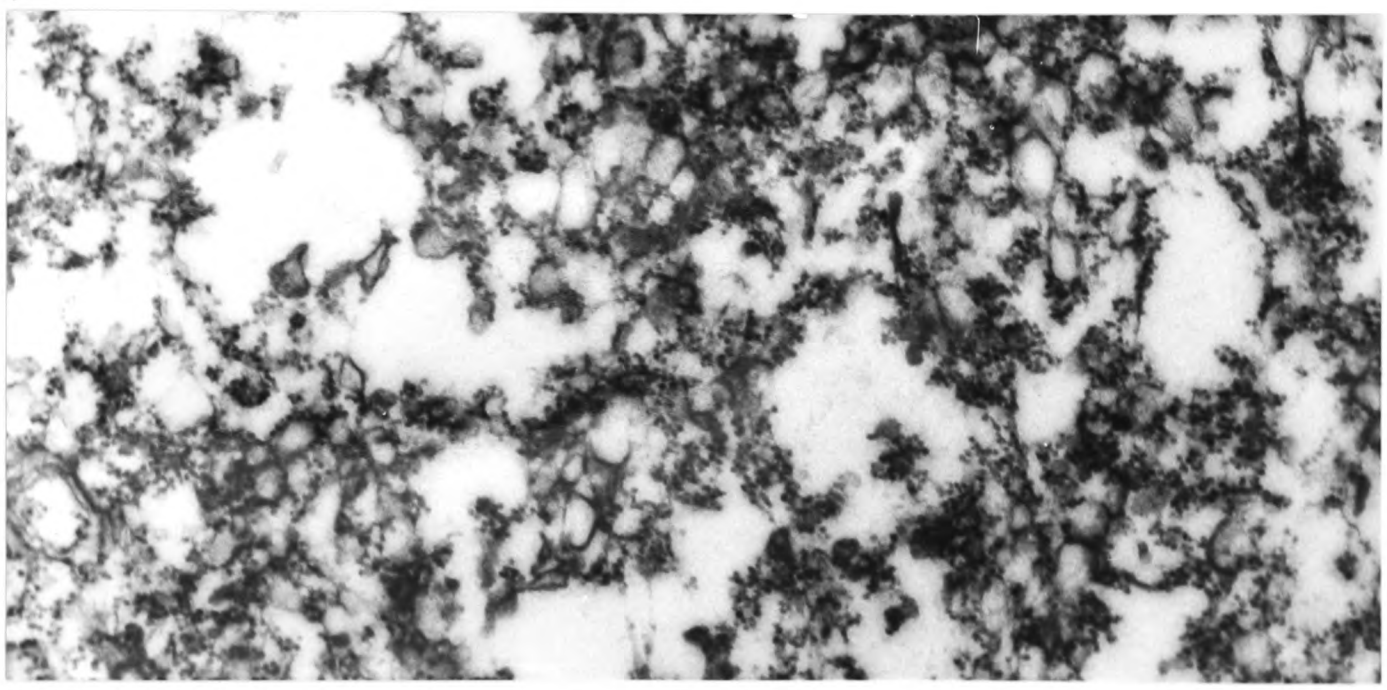
(a) Mitochondrial fraction x 32,000



(b) Fraction X x 40,000



(c) Fraction V (outside edge of pellet) x 26,000



(d) Fraction V (inside of pellet) x 40,000

III. RESULTS OF ELECTROPHORESIS

(i) General.

Electrophoresis on polyacrylamide gels of the soluble proteins from pea seedlings resulted in the successful separation of about fifteen visible components.

It was rather surprising that of the hundreds of different proteins which are present in every cell only 15 discrete fractions were obtained. There were occasionally a few more, the extra ones being feint bands which appeared between the others and only showed up in some separations. The identity of the bands is not known and it is possible that each band is composed of several proteins which have the same mobility. If there were proteins of a continuous range of mobilities after separation there would be just a smear along the gel. The appearance of sharp bands argues in favour of their individuality. However there is another factor which may result in proteins of very similar mobility running together and thus appearing as a sharp band. This is the tendency of protein molecules near a region of high protein density to run with the mass of the protein rather than to separate from it during electrophoresis.

When acrylamide gels were stained, the background between the bands also stained, and presumably this represented all the other proteins which were present in quantities too small to form a band.

Protein bands vary in intensity and sharpness. Although some diffuse bands are formed at the top of the gel, bands of high mobility tend to be more diffuse than bands of low mobility. The reason for this is that the front of the disc spreads more rapidly than by diffusion alone, if either the pH of the disc is lower than that of the buffer, or if the conductivity in the disc is higher than that of the buffer (Ornstein, 1964). The lower the mobility of the disc in the gel, the smaller will this effect be.

The protein bands obtained from the soluble proteins from pea seedling roots have not been identified. They cover a large range of mobilities but all are separated over a convenient distance on a 7.5% gel, Ornstein used a 7.5% gel, to separate plasma proteins. These proteins ranged from molecular weight of 69,000 and diameter 38 Å to molecular weight 1,300,00 and diameter 185 Å. The smaller of these after 25 minutes of electrophoresis had migrated 2.5 cms. into the fine pore gel and the larger one had just entered this gel. The mobilities of proteins on gels depend also on the net charge (Ornstein found that Y-globulin, although only 156,000 molecular weight and 44 Å in diameter did not enter the gel) but it is probable that the sizes of pea root proteins separated fall within the range: 60,000 - 1,400,000 molecular weight.

The Rf values for protein bands can be calculated, using the position of the tracker dye to mark the position of the front. But the bands have a characteristic appearance and the same band can be recognised on different gels even if slight differences in the electrophoresis have resulted in it moving a different distance into each gel. Therefore it is possible to compare a band on one gel with the equivalent band on another gel without using Rf values. (In results in the present study Rf values have occasionally been quoted.)

(ii) Regions of the pea seedling.

Proteins extracted from the different regions of the seedling (illustrated in Fig. 6) showed considerable differences between them. This was encouraging, since if there had been no difference between organs the chance of picking up differences between regions within one organ would have been small.

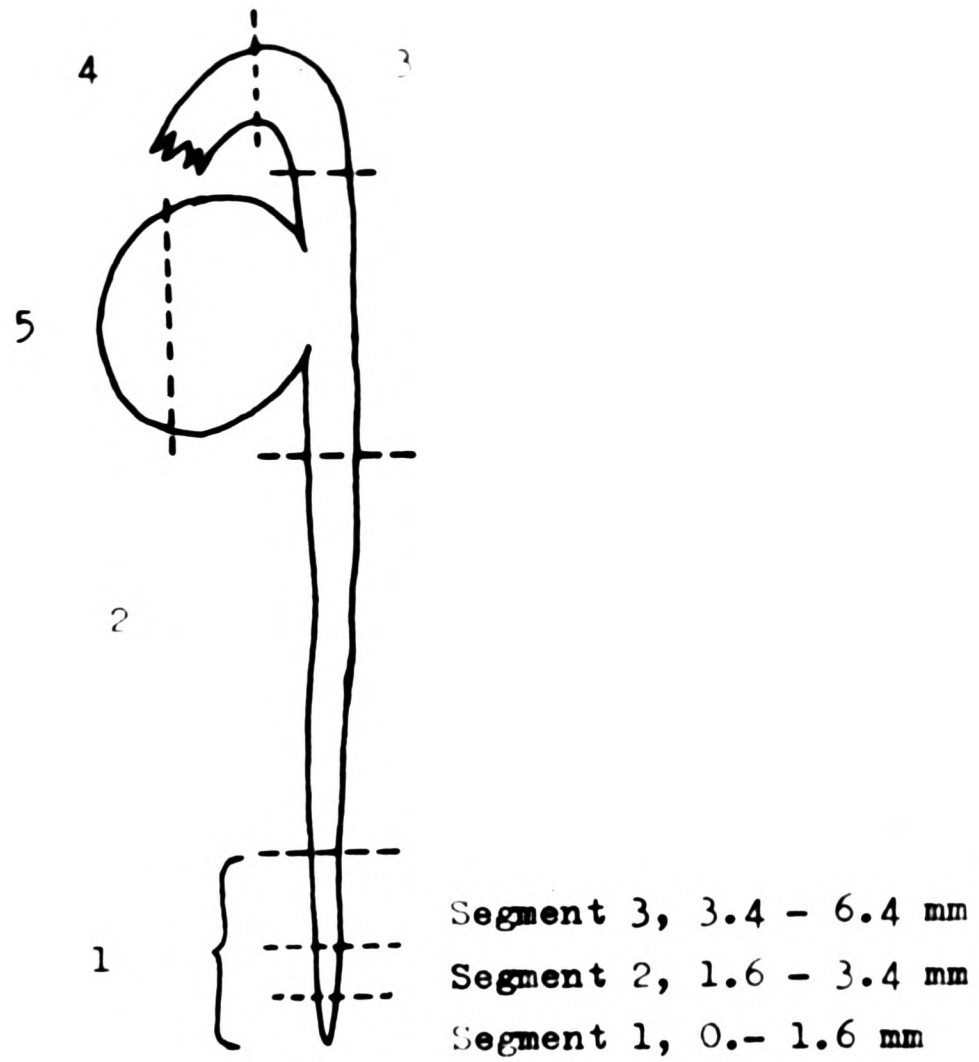


Figure 6. The different organs of the pea seedling from which proteins have been extracted.

1. The root tip
2. The proximal region of the root
3. Plumule stalk
4. Plumule
5. Cotyledon

The separations obtained from the root tip, root base, plumule, plumule stalk and cotyledons are shown in fig. 7. The scan of the cotyledon proteins showed one very strong protein band which was not present in the other regions. This may be a storage protein. In the other regions, in general the same major components were visible, but there were considerable differences in the relative intensities of the protein bands. In the centre region of the gels containing plumule and plumule stalk protein, for example, the same bands are present, but because of the differences in intensities of the bands, the gels have a quite different appearance.

When the differences in protein pattern between the different regions had been established, protein patterns from regions of the root tip were examined.

(iii) (a) Segment 1 (0-1.6 mm), 2 (1.6-3.4 mm) and 3 (3.4-6.4 mm). Results are shown in Figure 8.

Since the protein patterns from different organs showed mainly quantitative differences, it was thought that protein patterns from the regions of the root tip, if they differed, would show only minor quantitative differences. There were some minor changes in the relative intensities of the protein bands, showing quantitative differences but the most striking feature of these separations was the appearance of a strong protein band from the expanding and maturing root segments which was not present in the meristem. It was usually stronger from segment 2 (the expanding region) than from segment 3 (the maturing region). This protein band appeared in nearly all separations and was therefore thought to be a genuine new protein band and not an artifact of the method. The appearance of the new band suggested that there is one protein at least present in expanding cells, but which is not synthesised by meristematic cells. On some gels however there was a feint band in the position of the new band in the protein from segment 1.

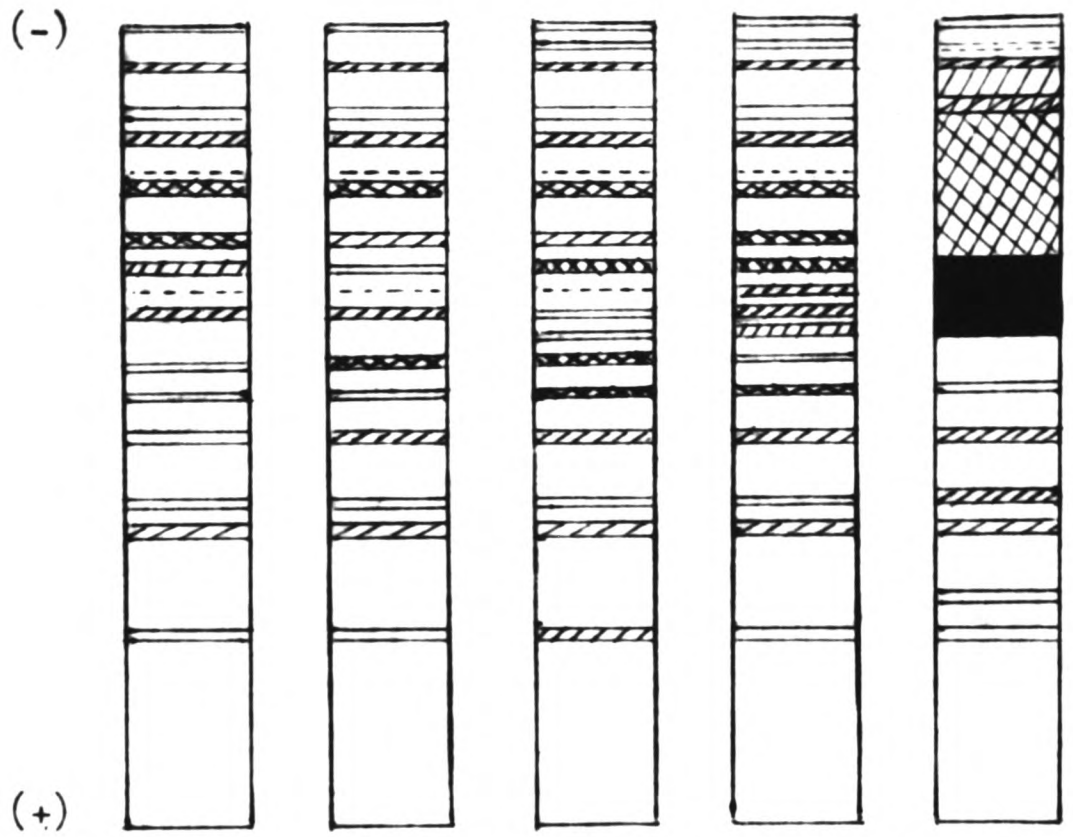


Figure 7. Protein bands from different regions of the pea seedling (i) the root tip, (ii) the proximal region of the root, (iii) the plumule stalk, (iv) the plumule and (v) the cotyledons.

Figure 8. Fractionation of soluble proteins from segments 0 - 1.6 mm, 1.6 - 3.4 mm and 3.4 - 6.4 mm of the pea root tip. Segments were homogenized in a sucrose medium buffered with tris/HCl and containing $MgAc_2$ (1 mM) and KCl (50 mM) and centrifuged to remove the cell particles. Electrophoresis was carried out on a 7.5% polyacrylamide gel in tris/glycine buffer at pH 8.9.

Figures 8 (a) and 8 (b) are scans made in the Chromoscan densitometer, of protein fractionated on different occasions. They illustrate the variation in results. Figure 8 (c) is a photograph of the gels; Figure 8 (d) a diagrammatic representation of the protein bands.

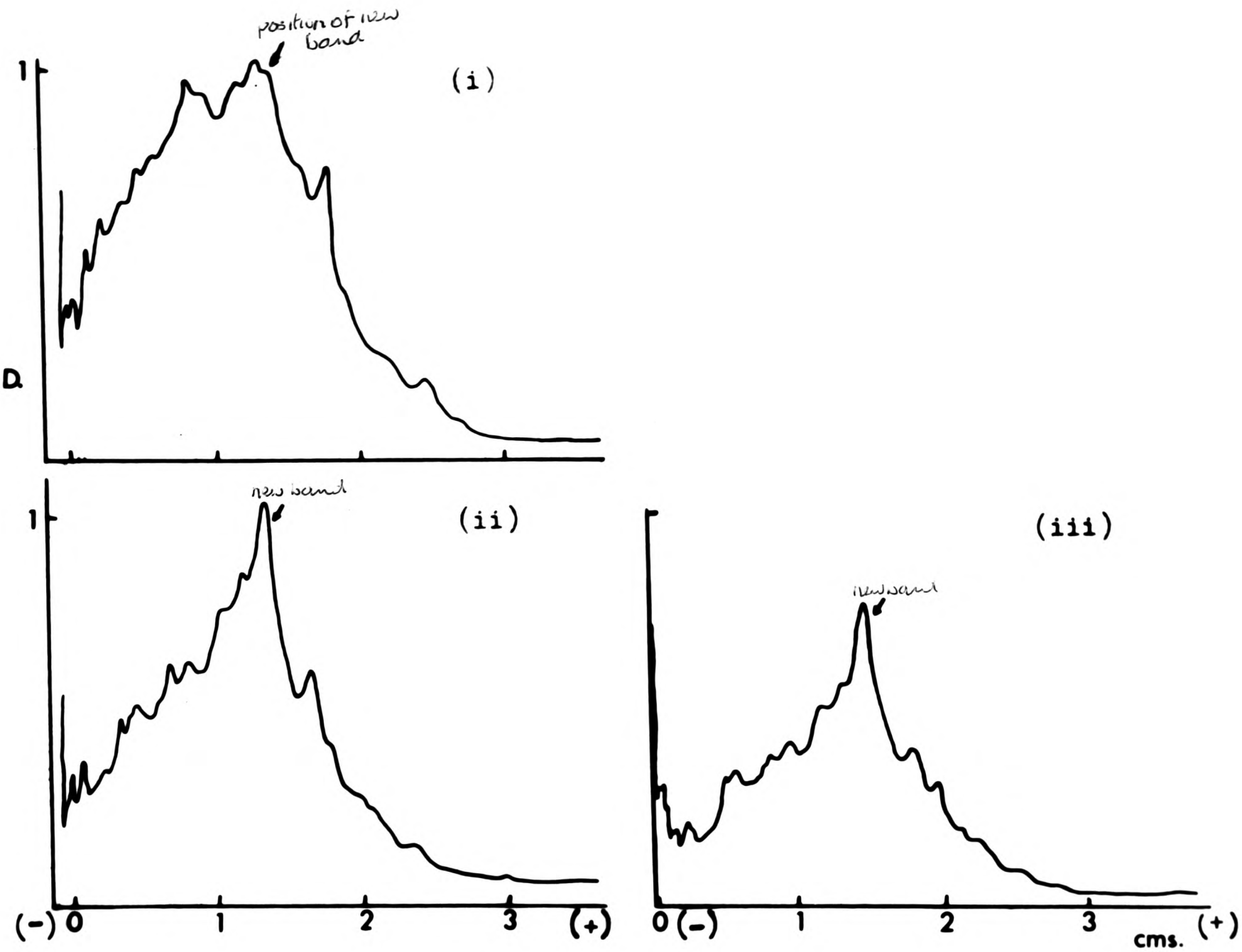


Figure 8 (a). Scans of protein from the pea root tip.
(i) Segment 1, (ii) Segment 2 and (iii) Segment 3.

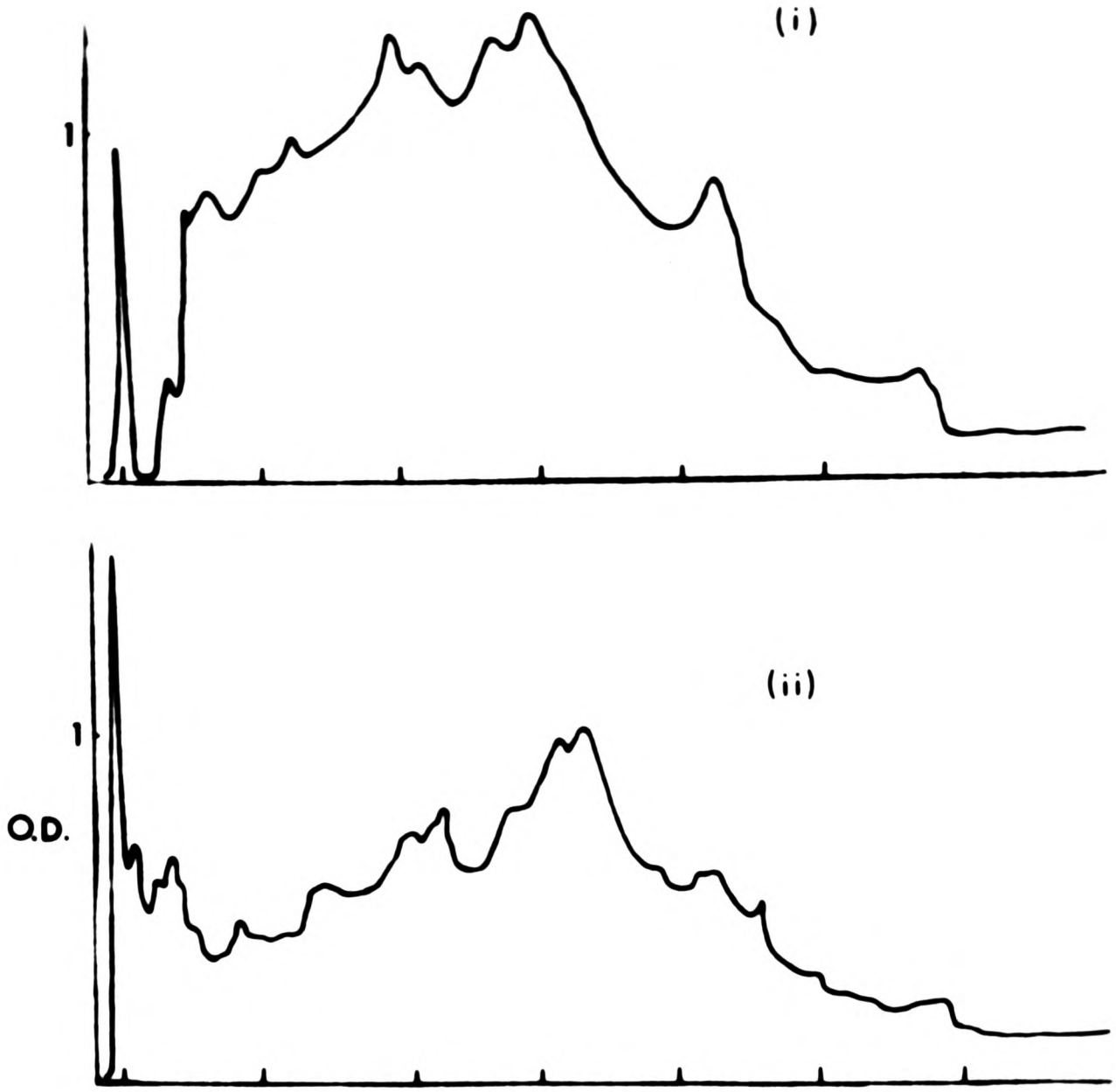


Figure 8 (b). Another scan of protein from the three segments of the pea root tip, to illustrate the variability of the method.

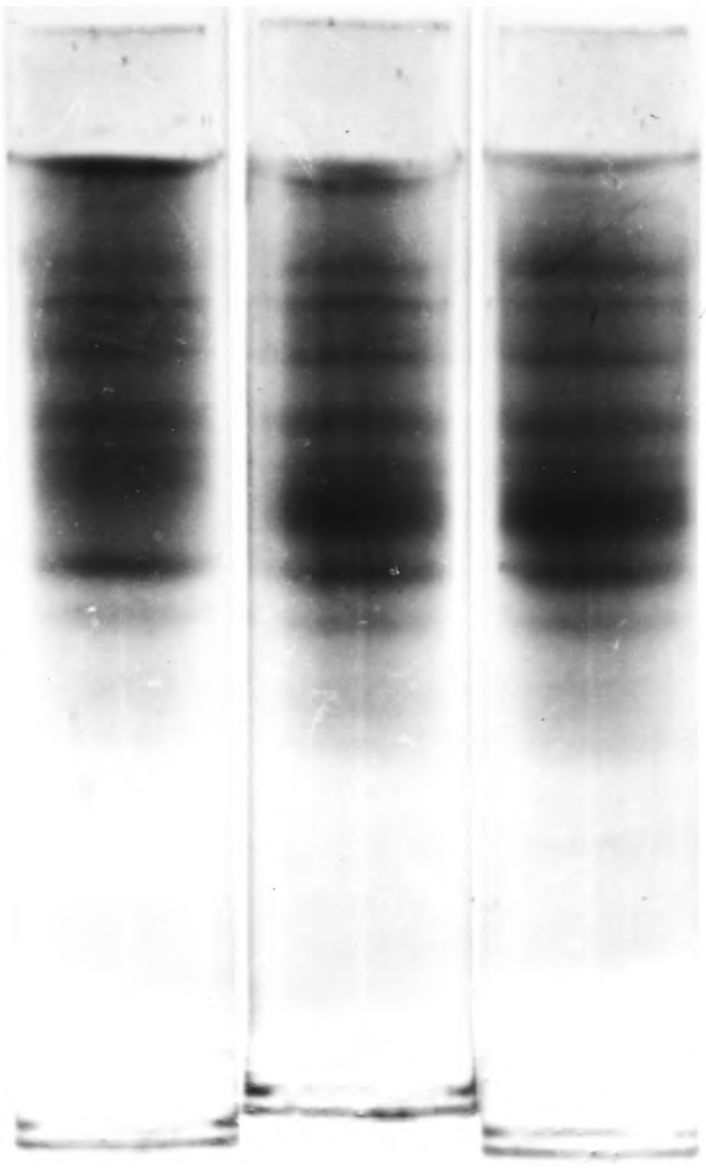


Figure 8 (c). Polyacrylamide gels showing protein bands from segments 1, 2 and 3 of the pea root tip.

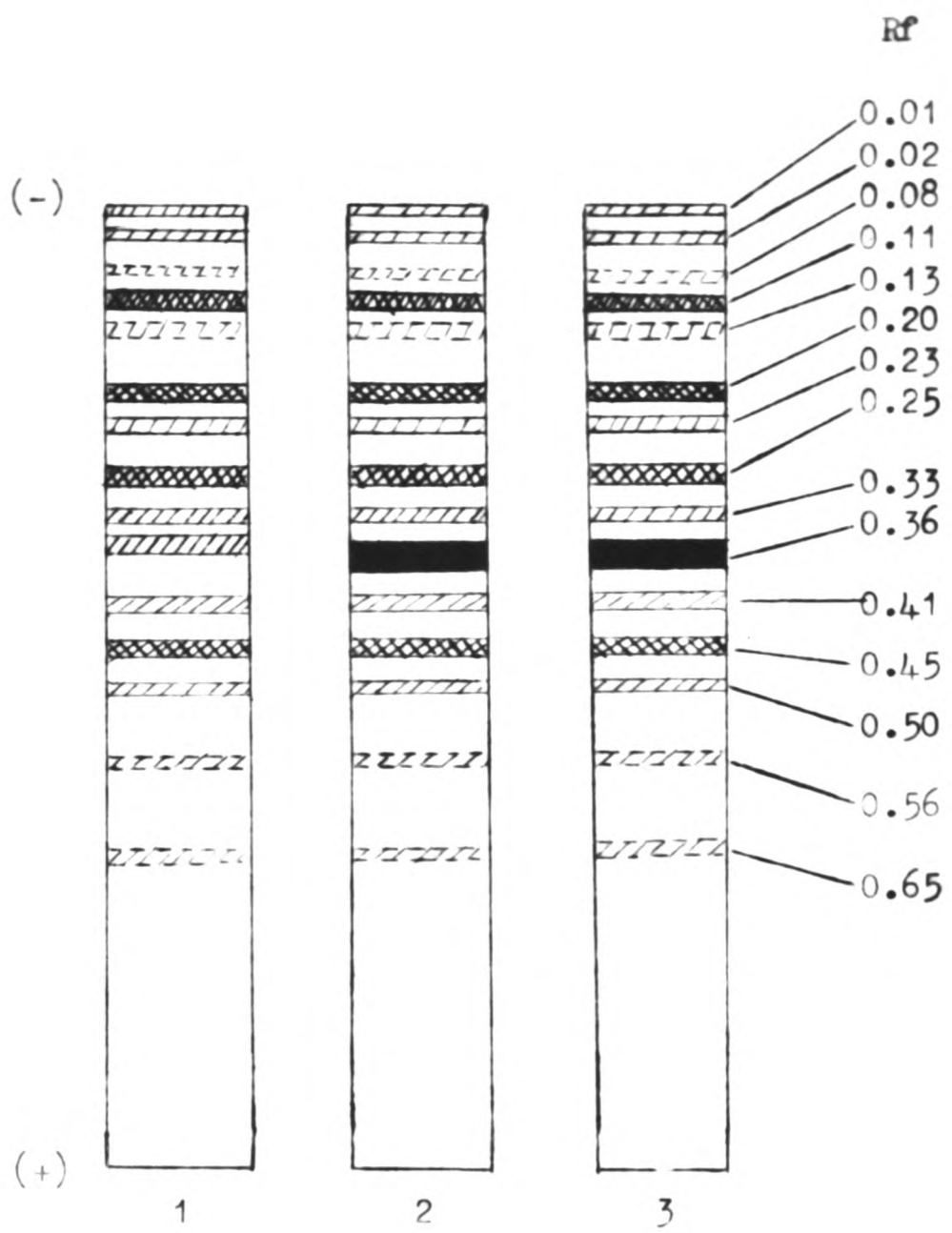


Figure 8 (d). Diagrammatic representation of the protein bands from segments 1, 2 and 3 of the pea root tip.

The other minor changes during differentiation involved changes in the intensity of protein bands, and besides the changes in the bands themselves there was a reduction in the background staining of the gel during differentiation. The meristem protein had quite a high background, and the protein bands were slightly blurred, but they became sharper and more distinct during differentiation. This is partly due to the reduction in background staining.

The changes which take place in the bands during development over the first 6 mms could be tabulated as shown in Table II.

Separations of the protein bands behaving as described in Table II have been obtained continually over the 3 year period, but the patterns were not always exactly the same, e.g. the new band on some occasions was less predominant than usual. This and other minor differences could sometimes be attributed to the peas or to some slight variation in technique, but not always. The same sample of protein was usually separated several times to make sure any differences were not due to the slight non-reproducibility of the method. Despite the variations the method was sufficiently reproducible for use as an analytical tool.

On the question of reproducibility it would be interesting to report here the results which were achieved for the first few months. The major part of the electrophoretic pattern was the same as that obtained in later separations, but ahead of the new band, two sharp bands appeared (Fig. 9). These bands, of fast mobility were so sharp that they had the appearance of salt fronts, and similar salt fronts were obtained when some other buffer systems were used (tris/citrate // tris/glycine). The stain at these salt fronts is taken up by proteins carried along by the front.

In one experiment, they did not appear, and in the tris/glycine buffer system, have not been obtained since. The reason for their appearance

Table II. The changes which take place in protein bands during differentiation.

Rf	Band Number	Change
0.01	1	No change
0.02	2	No change
0.08	3	Fades, almost indistinguishable in segment 3
0.11	4	Very sharp, no change
0.13	5	Becomes stronger
0.20	6	Fades
0.23	7	No change
0.25	8	No change
0.33	9	Sharper in segment 2
0.36	10	New band, trace in segment 1, strong in 2 and 3
0.41	11	Fades
0.45	12	Fades
0.50	13	Gets stronger
0.56	14	No change
0.65	15	No change

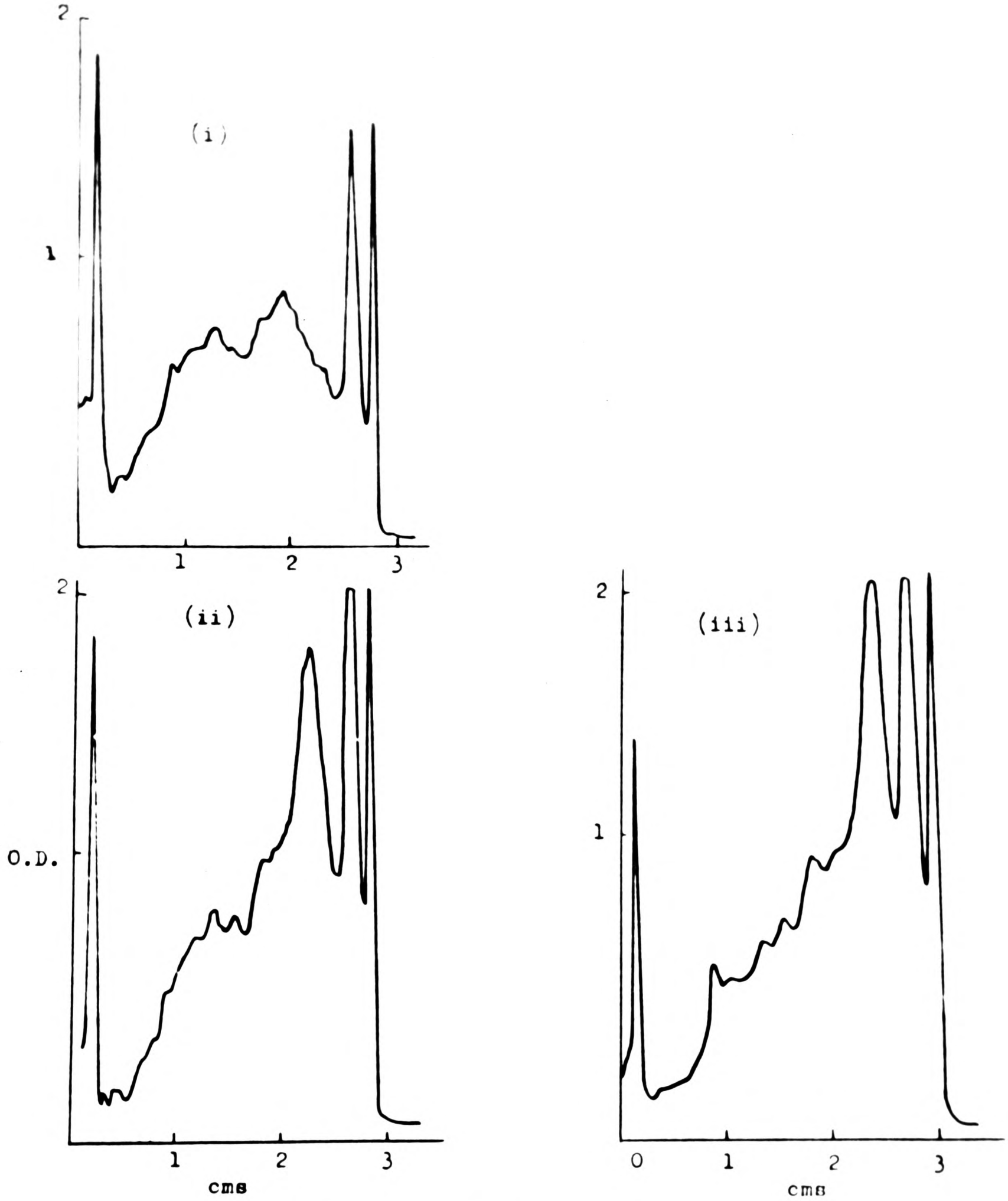


Figure 9. Protein from the three segments of the pea root tip. This was an early separation in which two salt fronts appeared.

in those early experiments was not discovered. They might have been due to the presence of an impurity in one of the chemicals, which was not present in subsequent batches of the chemicals.

Apart from the loss of these two sharp bands the only difference in the pattern was the appearance of a few feint bands in, and ahead of, the positions occupied by the salt fronts, which presumably had been previously masked.

Since the loss of the two salt bands the protein separations have been more or less reproducible except for one occasion. On this occasion the gel failed to polymerize properly, and the gels formed were very soft and flabby. Protein from the three segments was separated on these gels and when they were stained showed an unusual pattern. Although there were some feint bands behind and in front of them, most of the protein ran in two strong bands, one of which was hardly visible from the meristematic segment. So in this separation the difference between the segments had been exaggerated (Fig. 10).

(b) Millimeter segments.

When the differences between the three segments had been established the roots were then cut into smaller segments to find out more precisely where the new band was being formed. For this, segments 1 mm in length from the apex to 6 mm from it, were cut.

In the separations of protein from these segments (Fig. 11), the new band was present from the second segment. The new band appeared to change its mobility during differentiation. In the third millimetre segment it ran faster than it did in the second, but in segments 4, 5 and 6, it was slowing down again. This change in mobility was reproducible. The change in mobility during differentiation would explain the rather diffuse

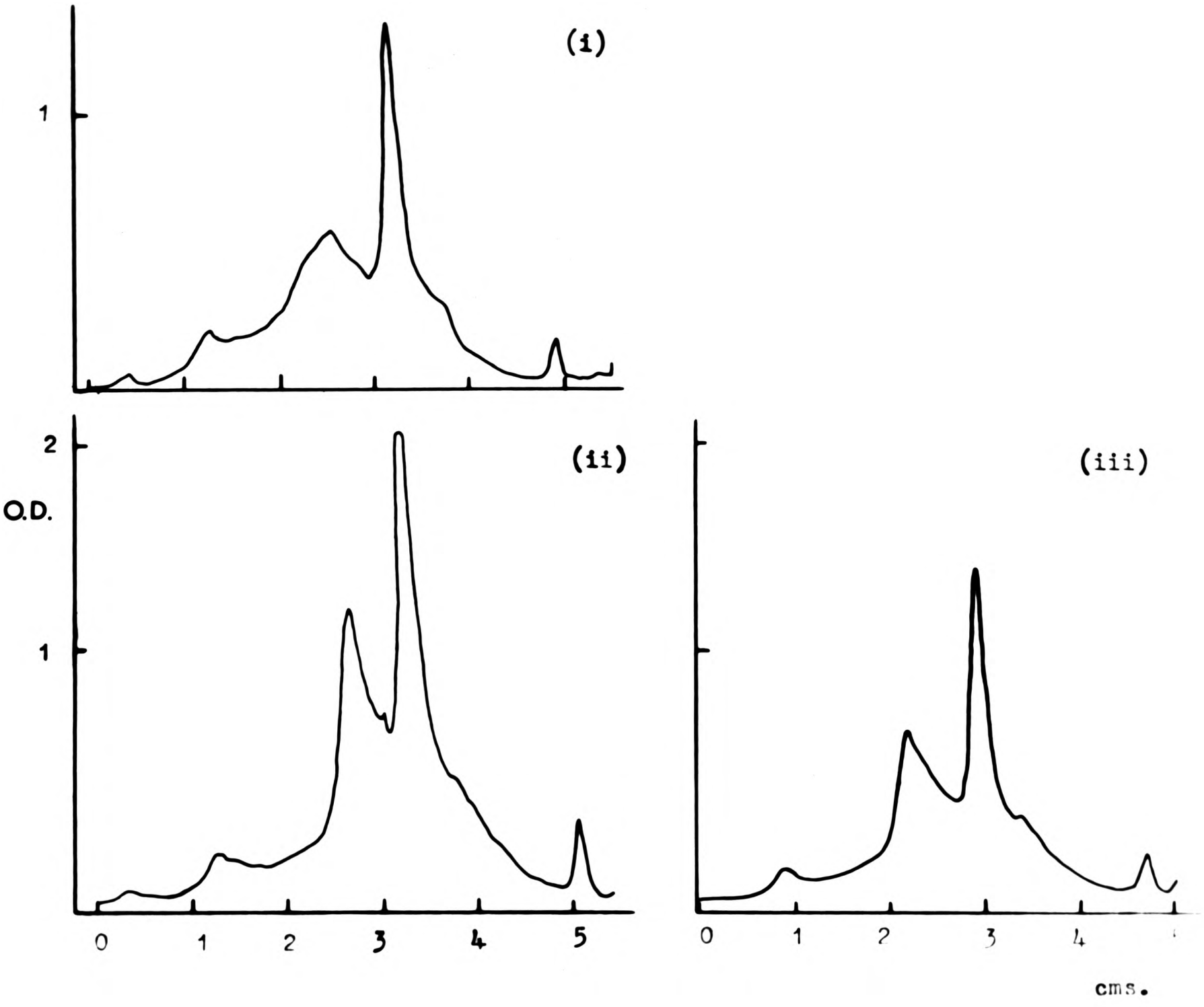


Figure 10. Proteins from segments 1, 2 and 3 fractionated on soft polyacrylamide gels.

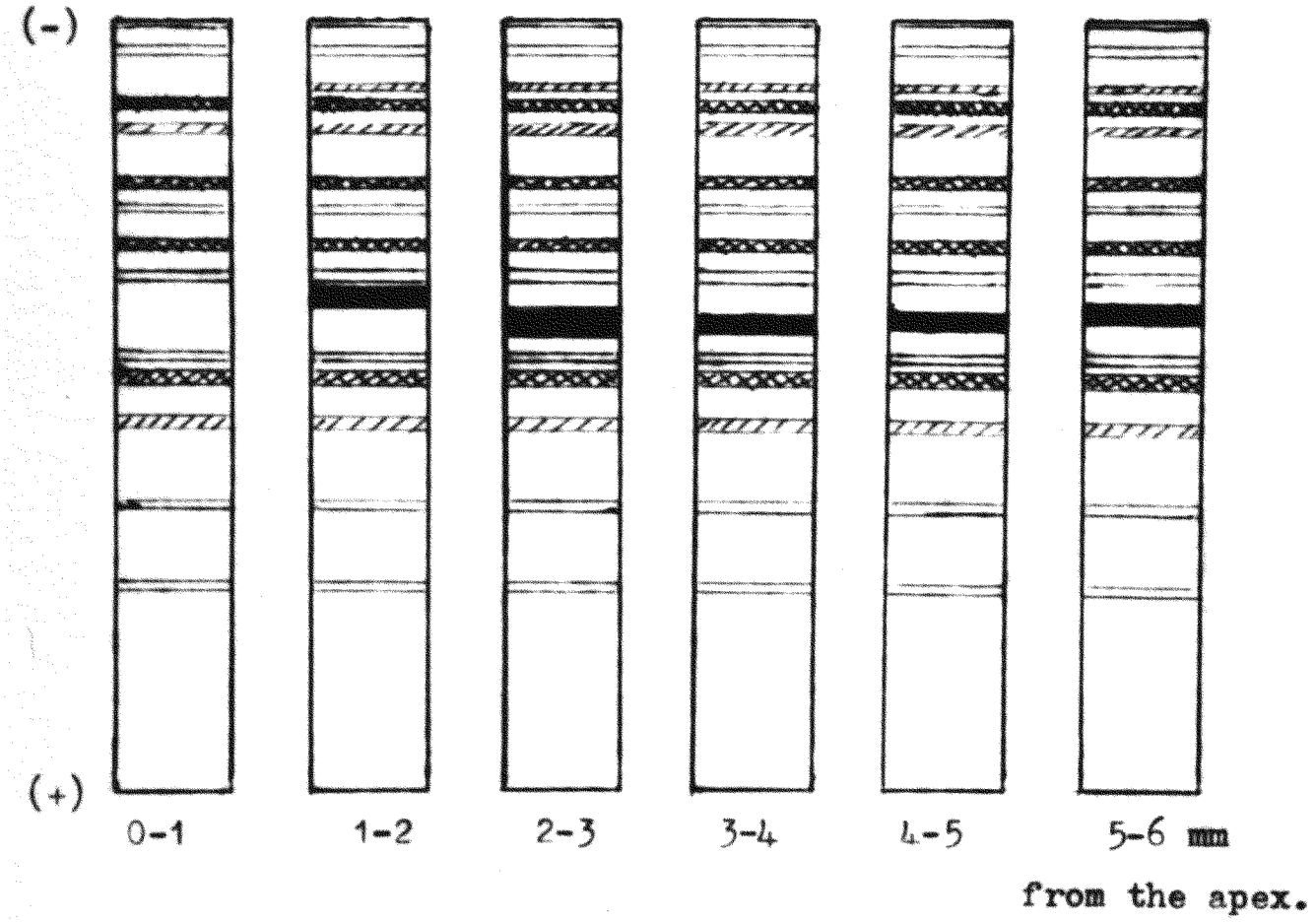


Figure 11.

Figure 11. Protein patterns from successive 1 mm segments of the pea root.

nature of the new band from the larger segments. Segments of lengths 0 - 1.6, 0 - 2.0, 0 - 2.4 mm were examined, and in the third of these the new band was wider presumably because it included more protein of faster mobility.

Steward et. al. (1965) separated protein from 1 mm segments of the root tip on polyacrylamide gels. They obtained a band in the position of the new band and it too had a similar change in mobility.

(c) Protein patterns from overlapping segments.

Segments of lengths 0 - 1.6, 0 - 2.1, 1.6 - 2.6, 1.6 - 3.4 mms have been examined to try to locate the position at which the new band begins to be formed.

There was no appearance of this band in the 0 - 2.1 mm segment, which suggested that it was synthesised towards the more mature end of the 1.6 - 3.4 mm segment, behind 2.1 mms. The new band did appear from a segment 1.6 - 2.6 mm so it seemed that the protein of which the new band is composed is synthesised between 2.2 and 2.6 mms from the apex. However, in the previous experiment the band was present from a segment 1.0 - 2.0 mms and there was occasionally a trace of it in the segment 0 - 1.6 mms. This suggests that in different roots the new band is formed at slightly different distances from the apex, or that the protein is being synthesised very slowly by meristematic cells and the rate of synthesis increases enormously when the cells begin to expand. This protein band from segment 1.6 - 3.4 mm will, however, be referred to as the 'new' band.

(d) Root cap proteins.

To find what proteins were contributed to the pattern from the meristem, by the root cap, protein was extracted from a large number of root caps and used for electrophoresis. No protein bands stained in the

gel, nor was there any difference in pattern between the 0 - 1.6 mm segment and the 0.5 - 1.6 mm segment, from which it was concluded that protein from the root cap cells contributes little if anything to the pattern for the meristematic segment.

(iv) Comparison of the tip with older regions of the root.

To find out if the pattern obtained from the 6 mm region represents the mature protein pattern, or whether there are further changes as cells age, protein was extracted from successive 7 mm root segments cut from the tip to the base of a 35 mm root. Results are shown in Figure 12. No new protein bands appeared, nor did any disappear, but there were small, gradual changes in some bands to the base of the root. Bands 6 and 8 decreased, while 13, 15 and 16 increased in intensity, and a change at the top of the root results in two bands becoming further separated. (The new band region was particularly prominent in this separation.)

The result does not agree with the suggestion by Morris (1966) that the protein complement at 6 - 10 mms represents the mature state and thereafter remains constant.

It was considered in the introduction that cells from the base of the root might have a different history from those at the apex, but no abrupt change in the protein pattern which might have indicated the boundary, was found.

It may be concluded that the proteins in pea root cells at differen stages of differentiation can be fractionated on polyacrylamide gels, and although much of the protein is polydispersed, fifteen or so discrete bands appear. During differentiation changes take place both in the poly dispersed proteins and in the bands. These are mainly quantitative

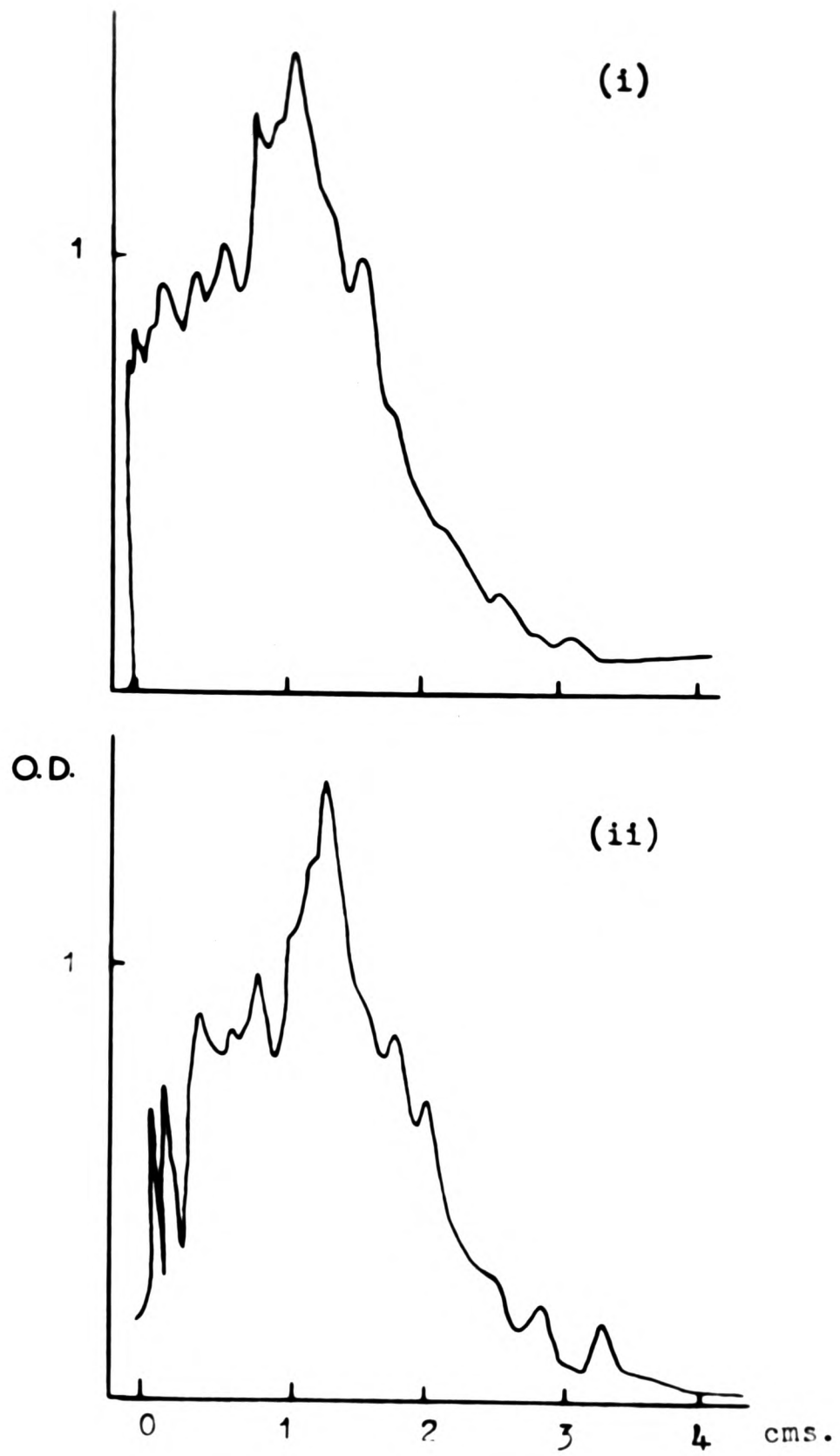


Figure 12. Proteins from the pea root tip and a more basal segment (21 - 28 mm).

and continue to the base of the root, but there is one qualitative change, a new strong protein band which develops during expansive growth and remains in the cells through maturity.

(v) Varied electrophoresis conditions.

When the method of electrophoresis described in section 11 (i) had been established and was working reproducibly, some of the conditions were altered to find out how the protein patterns produced in these conditions compared with those normally obtained. The experiments are described in sections according to which part of the normal procedure was altered. The control in each case refers to the gel used for electrophoresis under normal conditions. The protein used for all these experiments was obtained from segments 1, 2 and 3, so that the comparison between segments could be made under the changed conditions.

(a) Different gels.

Storage. Gels were usually allowed to stand for 1 hour before they were used for electrophoresis. The effect of storing the gel for longer periods before use was examined. If the results obtained on older gels were as good, it would have meant that large numbers of gels could have been prepared at one time, for use on subsequent days.

The protein pattern obtained on gels 1 and 2 days old showed bands which were slightly sharper than the controls, but on gels which had been stored for 4 days, the bands were less distinguishable. The slight increase in resolution after the shorter period of storage may have been the result of the gels hardening during storage: a process which probably involved a decrease in pore size and which would account for the sharpening of the bands. Whatever the process, it resulted in a general change in the properties of gels during storage, leading eventually to one which was

inferior to the control. For this reason, gels were always prepared 1 hour before use.

Spacer gel. The importance of a spacer gel according to Ornstein (1964) and its unimportance according to Raymond (1964), was discussed in the introduction. Its importance in the present system was examined by comparing protein patterns obtained on gels with and without a spacer gel.

The protein patterns in the two instances were indistinguishable. However, since preparing the spacer gel was part of the routine, and because it served to trap large proteins and any ribonucleoprotein particles which had failed to sediment in the last centrifugation (the surface of the spacer gel usually took up some protein stain), its use was retained.

Slow polymerization. Most workers arrange for polymerization to take place in about 40 minutes. Polymerization in my system took 2 - 3 minutes. If these rapidly polymerized gels were held against the light, refractile boundaries could be seen running through them, and it was thought that these might be the result of the rapid polymerization. Polymerization was slowed down either by (1) the addition to the monomer solution of potassium ferricyanide (final concentration 25ug/ml) or (2) allowing the gels to polymerize at $0^{o}C$ or (3) mixing the two catalysts ($(NH_4)_2SO_4$ and TEMED) and allowing them to stand for 1 minute before adding them to the monomer solution.

In all cases the refractile boundaries in the gels could again be seen, and since they had no adverse effect on the fractionation or scanning of gels, they were ignored.

Results from (1) showed the protein pattern to be indistinguishable from the control. Results from (2) and (3), however, were different. They both produced diffuse bands which suggested that these gels had a larger

pore size. From this it was concluded that the polymerization time was not critical, but that gels may polymerize to a different degree under different conditions.

(b) Different buffers.

So many workers, including Poulick (1957), Chang et. al. (1962), and Ornstein (1964), had recommended the use of a discontinuous buffer system, that a comparison was made of protein patterns obtained in continuous and discontinuous buffer systems.

The following buffer systems were used: Tris/citrate in the gels, boric acid/sodium hydroxide in the elctrode vessels (Poulick, 1957); tris/citrate in gels, tris/glycine in the electrode vessels; and a continuous tris/citrate buffer system. The pH's of these buffers were as follows: tris/citrate pH 8.65, boric acid/sodium hydroxide pH 8.65 and tris/glycine pH 8.9. The control was a continuous tris/glycine system pH 8.9.

During electrophoresis in the Poulick buffer system, there was a lower voltage for the same amount of current as in the control, but the run was completed in the same length of time. The protein pattern at first glance seemed to be completely different from the control, but when compared closely it was found that the same protein bands were present but they had been further spread out in the discontinuous buffer system (Figure 13). The increase in band 10 (the new band) in this separation was striking, and changes in the relative intensities of the other bands could also be clearly seen.

In the buffer system with tris/citrate in the gels, and tris/glycine in the electrode vessels the protein pattern obtained was the same as that obtained in the continuous tris/glycine system. In the continuous tris/citrate system, however, although the voltage and current

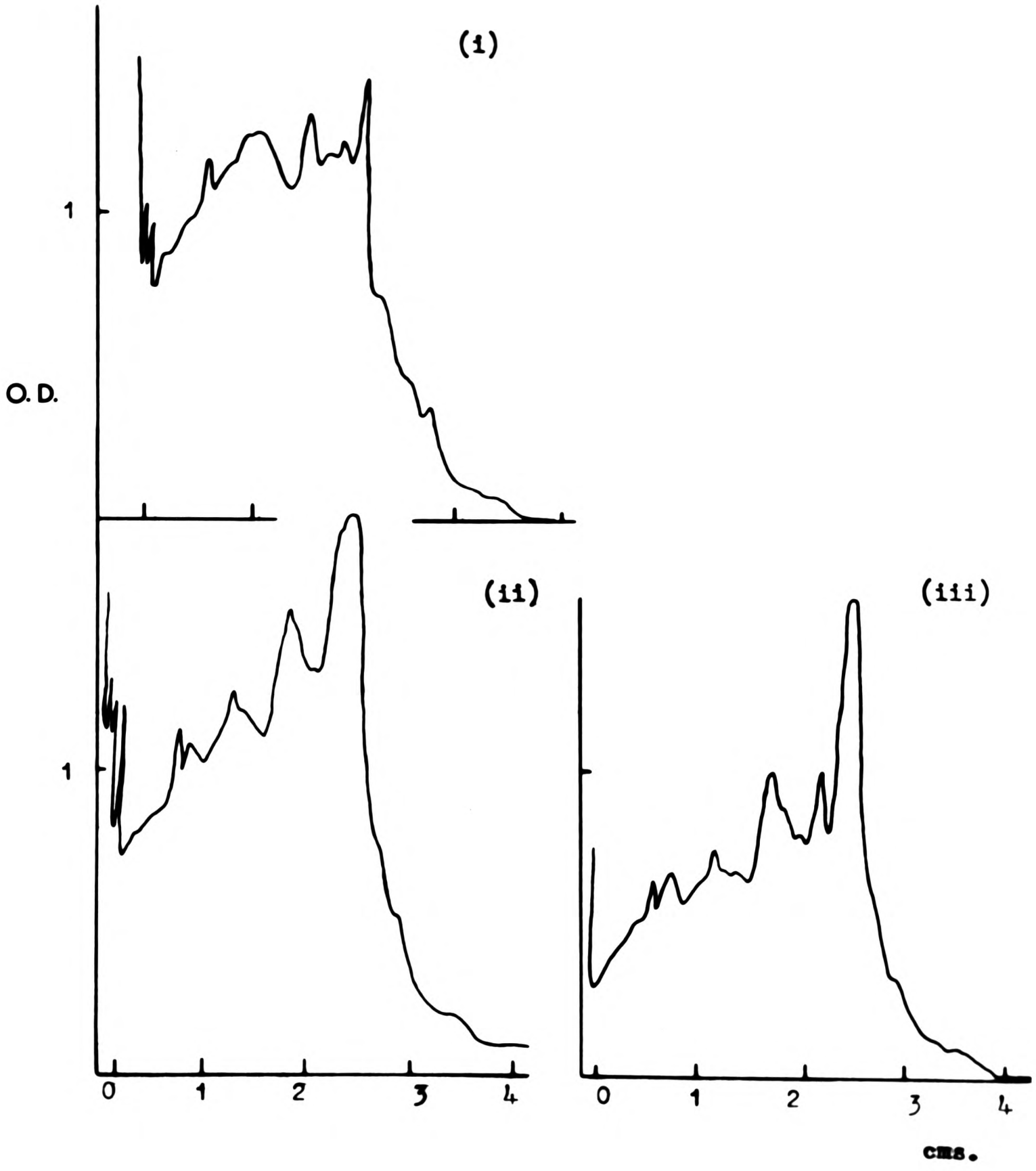


Figure 13. Electrophoresis in a discontinuous buffer system tris/citrate in the gels, boric acid/sodium hydroxide in the electrode vessels.

had been almost the same as in the controls, the protein had moved less than one centimetre into the gel, and had not separated into bands.

It has not been taken into account that the proteins when applied to the gel were in a medium which was itself buffered with tris/HCl. The normal buffer system may therefore be described as discontinuous, but because the mobilities of the buffer ions ahead of, and behind the proteins were the same, neither the sharpening effect described by Ornstein nor that described by Poulick would operate.

(c) Different pre-treatments of the protein.

Storage. Soluble protein samples were not usually prepared and fractionated on the same day. They were stored frozen (at -20°C) until they were needed. A comparison of proteins which had been stored in this condition with freshly prepared protein showed no difference in pattern after fractionation. In fact it was found that protein could be stored at this temperature for 6 months or longer with no apparent change in the subsequent pattern of bands.

Extraction. Proteins were normally extracted in a sucrose medium with tris/HCl buffer at pH 7.6 and containing magnesium and salt (KCl) which helped to maintain the integrity of ribosomes and stabilize other subcellular structures. It was thought that the removal of these from the extraction medium would result in a dissociation of subcellular components with the possible release of more proteins into the soluble fraction.

Proteins extracted in a medium without salt or magnesium produced slightly diffuse protein bands on electrophoresis, compared with the control but no extra protein bands could be seen.

The extraction of proteins in a different buffer (KH_2PO_4/NaOH, instead of tris/HCl) either at the same pH (7.6) or at a lower pH (6.4) did not affect the protein pattern.

Purification. If the soluble proteins in the supernatant were purified by precipitation with ammonium sulphate, and dialysis in tris/glycine buffer prior to electrophoresis, the protein bands obtained were diffuse compared with the control. The resolution of the central bands in particular, was lost, and the background staining was somewhat darker. The same diffuse pattern was obtained if the supernatant was dialysed (without precipitation) prior to electrophoresis. This finding that dialysis resulted in a loss of resolution of subsequent protein bands agreed with results obtained by Steward and Barber (1964).

Whatever changes were made in the electrophoresis conditions, the same differences in pattern between the meristem proteins and proteins from expanding and mature tissue were found. In the tris/citrate//boric acid/ sodium hydroxide buffer system the differences were more clearly seen, but the method described in Section 11 (i) continued to be used routinely.

(vi) Cultured segments.

Cultured segments were studied for the reasons described in the introduction.

The biochemical changes which take place in cultured root segments have been investigated (Robinson and Brown, 1954; Loening, 1965; Vaughan, 1965). It has been found that, although there is no net increase in protein, protein turnover is taking place. It was therefore of interest to find out what changes were taking place in the protein complement of cells during culture, compared with those already found to occur during differentiation.

(a) Methods.

7 mm pea root tips were excised and 20 placed in each 50 ml conical flask, together with 5 ml of a 2% sucrose solution. The flasks were

stoppered with cotton wool plugs and agitated on a rotary shaker rotating at 80 - 120 revs/min. Some root tips were also cultured in water for comparison. To study the effect of actinomycin D, the antibiotic (5 - 20 ug/ml) was added to the sucrose solution.

(b) Results.

The results are summarized in Table 3, which shows that if segments were cultured for long enough (24 hours) in either sucrose or water, the protein pattern became like that shown in Figure 14, (b) and (c). This is the pattern described as 'changed' in Table 3. Compared with the normal protein pattern from the fresh root segments, there was a large increase in the intensity of some of the components of low mobility, (Rf = 0.1), relative to the other bands, and also an increase in the protein of the new band and the bands running just behind it (Rf = 0.36).

These changes took place only in the longer segments (1.8 and 3.4 mm) cut from the expanding and maturing regions, or in the 7 mm tip. They did not occur in smaller segments (1 mm in length) from any region of the root tip, nor did they occur in the 1.6 mm apical segment. In these cases there was a blurring of the protein pattern with just a slight increase in the proteins of Rf = 0.1.

It was rather surprising to find that the same change, during culture, occurred in the 7 mm root as in the smaller segments. It could have been argued that the smaller segments were beginning to autolyse and the change in pattern attributed to this factor, but 7 mm root tips are able to support themselves in 2% sucrose and, after a time lag, to resume growth. During culture the increase in the new band and adjacent bands took place between 12 and 16 hours, and that of the slower moving bands took place between 16 and 24 hours.

Table III

Segment	Solution	Time	Inhibitor	Result
7mm tip	Water	12 hr		Bands in the Rf 0.1 and 0.36 regions have slightly increased intensity.
		24 hr		Changed
	Sucrose	12 hr		Pattern as after 12 hr in water.
		16 hr		New band region very prominent.
		24 hr		Changed
		12 hr	2 µg/ml	As controls after the same lengths of time.
		16 hr	2 µg/ml	
		24 hr	20 µg/ml act. D	
Seg. 1, 2 + 3	Water	24 hr		Changed
	Sucrose	$2\frac{1}{2}$ hr		Less change in seg 2 than in 1 or 3.
1mm tip	Sucrose	$5\frac{1}{2}$ hr		New band had slightly increased mobility.
		19 hr		Bands very diffuse.

Table III. The changes which take place in the protein pattern when segments of the pea root tip are cultured in various media for different lengths of time.

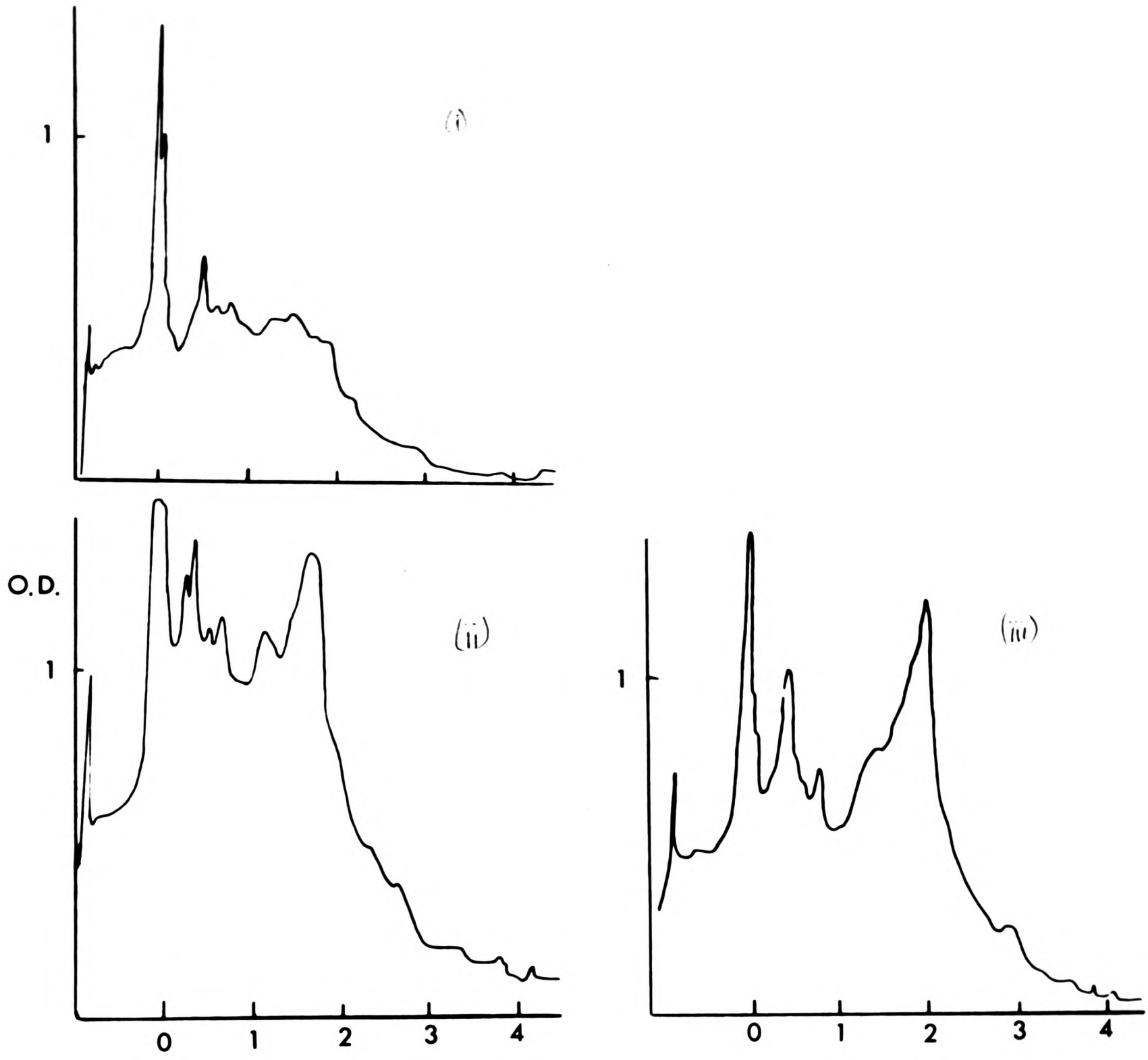


Figure 14. Protein from segments 1, 2 and 3 of the pea root tip, excised and cultured for 24 hours in water.

The presence of up to 20 ug/ml actinomycin D in the culture solution did not affect this change of pattern.

The changes in pattern were unlikely to be due to bacterial protein for two reasons. Firstly, the homogenization procedure would not break the bacteria: they should therefore have been removed by the centrifugation. Secondly, the tip segment had a different pattern.

IV. ENZYME STUDIES

(i) Introduction.

It has been shown that many enzymes are heterogeneous, and that the different components separate on electrophoresis so that by staining a gel for the activity of one enzyme, several bands of coloured reaction product are formed. These 'isozymes' and their relative amounts differ with the organism, or even with the tissue.

Markert and Moller (1959) have shown that there are species specific and also tissue specific isozymes of lactic dehydrogenase. Further it has been shown (Moore and Villee, 1963) that different isozymes are present at different stages of development of one tissue. Malic dehydrogenase from sea urchin eggs produces different numbers of bands depending on the stage of development. Unfertilized eggs have 5 bands, small blastomeres have 3 bands while large blastomeres have only 2 bands of activity.

It has also been shown that there are more isozymes in induced that in uninduced bacteria (Appel and Alpers, 1965). Uninduced bacteria produced one band of β-galactosidase but induced cells produced 7 bands, one of which was in the same position as the uninduced band, while the rest were slower moving. The new bands consist of enzyme molecules which are either larger or which have less charge. The suggestion was made that the molecules contained different proportions of different polypeptide chains.

It has been stated that the number of bands depends on the tissue. Thorne, Grossman and Kaplan (1963) obtained 6 separable forms of malic dehydrogenase from an enzyme preparation of pig heart, after electrophoresis on starch gel, and they found differences between mitochondrial

and cytoplasmic forms. A difference in pea seedlings between mitochondrial forms of malic dehydrogenase was established on kinetic data, by Davies (1961).

The significance of the multiple bands obtained on electrophoresis is debatable. McKinley-McKee and Moss (1965, a and b) showed that although they obtained several bands of enzyme activity (alcohol dehydrogenase from liver) these were not different proteins, but the same enzyme forming complexes with coenzymes or other nucleotides. These should not therefore be called isozymes. On the other hand, Thurman, Palin and Laycock (1965), obtained several bands (L - glutamic acid dehydrogenase from Vicia seeds) which they claimed were isozymes.

The object of this study was not to establish whether or not the different bands represent isozymes, but to find out if any new components appeared during the development of the pea root, or if there were any changes in the relative amounts of the enzyme components as there had been with the protein bands.

(ii) Methods.

Following electrophoresis, the gels were placed in an incubation medium containing substrate, coenzyme and a suitable coupling dye.

Test for acid phosphatase. The methods of (1) Kates and Goldstein (1964), and (2) Grogg and Pearse (1952) were compared.

(1) 20 mg 1-naphthyl acid phosphate
200 mg tetrazotised diorthoanisidine
in 100 ml 0.02 M acetate buffer at pH 4.6 for 46 minutes.

(2) 14 mg α-naphthyl phosphoric acid
10 mg o-anisidine tetrazolium salt
in acetate buffer at pH 5.0.

Test for dehydrogenases. The method of Thorne et. al. (1963) was used.

DPN	0.7 mgms/ml
phenazine methosulphate	0.05 mgms/ml
nitro blue tetrazolium	0.4 mgms/ml
substrate	5.0 mgms/ml

in tris chloride buffer at pH 8.4 (0.1 M).

When the substrate used was an acid e.g. malic acid or glutamic acid, it was first dissolved in water and neutralized with NaOH before being added to the incubation mixture.

After electrophoresis at room temperature the gels were removed from the glass tubes and placed in corked tubes containing 3 mls of the incubation mixture. These were incubated at 37^{o}C until the bands appeared.

Preparation of soluble enzyme fraction from mitochondria.

55 of each of the 3 segments of the pea root were homogenized separately with 1 ml of homogenizing medium containing 0.4M sucrose, 0.005 M EDTA in 0.1 M phosphate buffer at pH 7.4.

The tissue was macerated at 1000 rev./min. until all the cells were broken, and centrifuged for 10 mins. at 800 x g. This removed the debris. The supernatant from this spin was contrifuged for 25 minutes at 10,000 x g. The precipitate was resuspended in homogenizing medium and re-centrifuged at 10,000 x g for 25 minutes, and the supernatant from this discarded. The mitochondria were disrupted with 0.5 ml phosphate buffer (0.1M) with 0.0025 M EDTA and 0.1% O·C 45, a non-ionic detergent. This was contrifuged at 100,000 x g for 25 minutes. The supernatant was dialysed overnight against 400 ml of 0.1 M phosphate buffer, at least 2 changes, with mercaptoethanol. The protein was then used for electrophoresis.

(iii) Results.

(N.B. The term 'protein band' is reserved for bands which appear on gels stained for total protein with Amido Black.)

Most of the enzymes tested for showed more than one band of enzyme activity.

Acid phosphatase. No activity of this enzyme was detected in the soluble fraction, but some activity was found to be associated with the particulate fractions.

Malic dehydrogenase. This was compared from the three segments (Figs. 15, a and b). There were four bands of enzyme activity in each segment, but the relative intensities of these bands were different in the three segments, i.e. they change during differentiation.

The fastest moving of these (4) was comparatively well defined in segment 1, and became feinter in segments 2 and 3. The bands behind this one, (bands 1 and 3) increased in activity during differentiation, and band 2 remained about the same intensity, although it appeared to have increased its mobility slightly in segments 2 and 3. The background staining decreased with the age of the cells in the same way that the background on the amido black-stained gels decreased. This resulted in the bands from the third segment being better defined.

There was also some enzyme activity at the surface of the fine pore gel. This was shown by scanning the gel at 5 minute intervals as the colour was developing. The change in refractive index at the surface of the fine pore gel always produced a peak, but in this case it increased in height with time, therefore must have been due to enzyme activity.

Sawaki et. al. (1965) have found 3 bands of activity of malic dehydrogenase in protein extracted from rat tissue and separated by electrophoresis on cellulose acetate paper. By incubating the paper

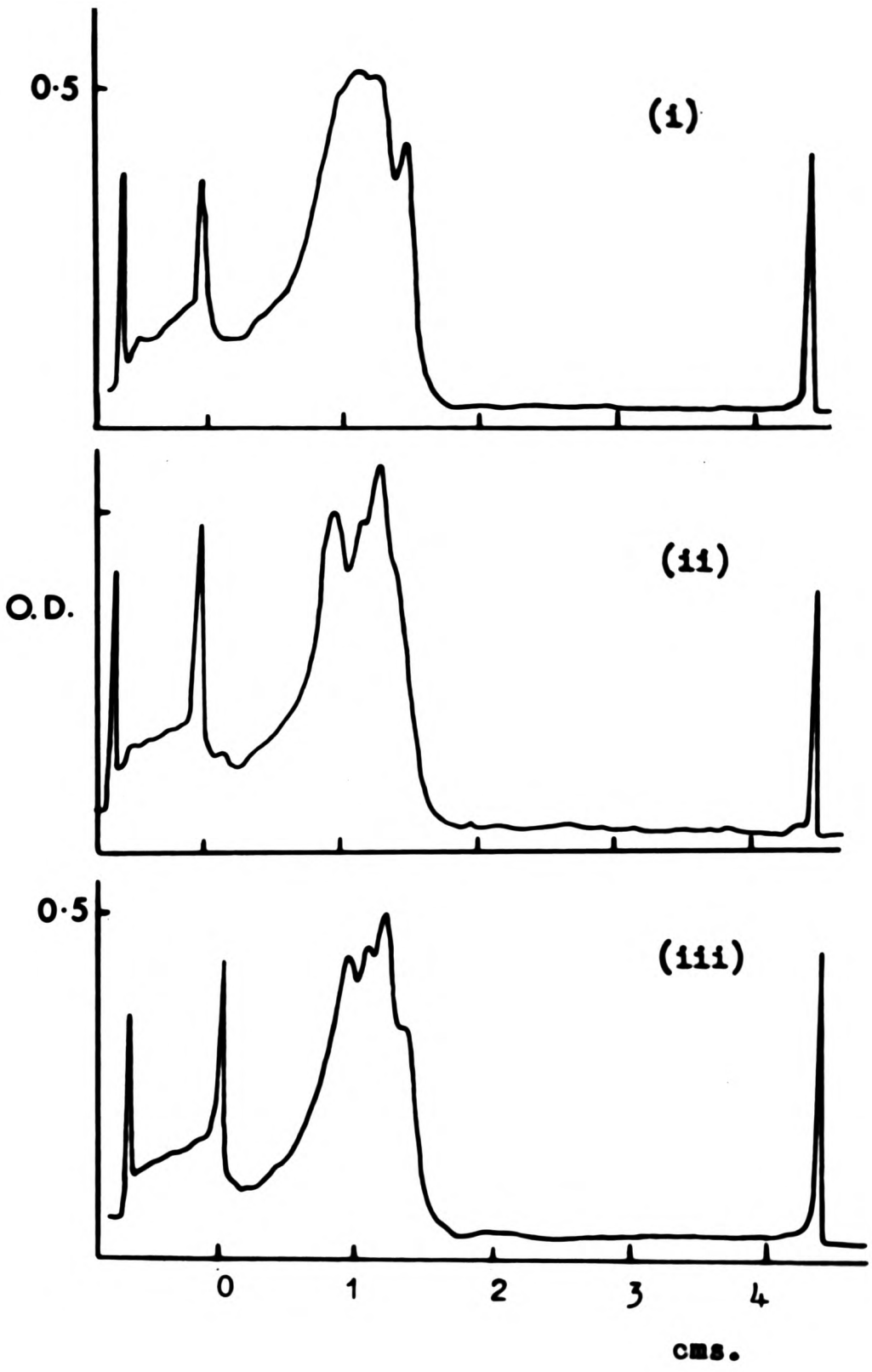


Figure 15.(a) Isozymes of malic dehydrogenase from segments of the pea root tip.

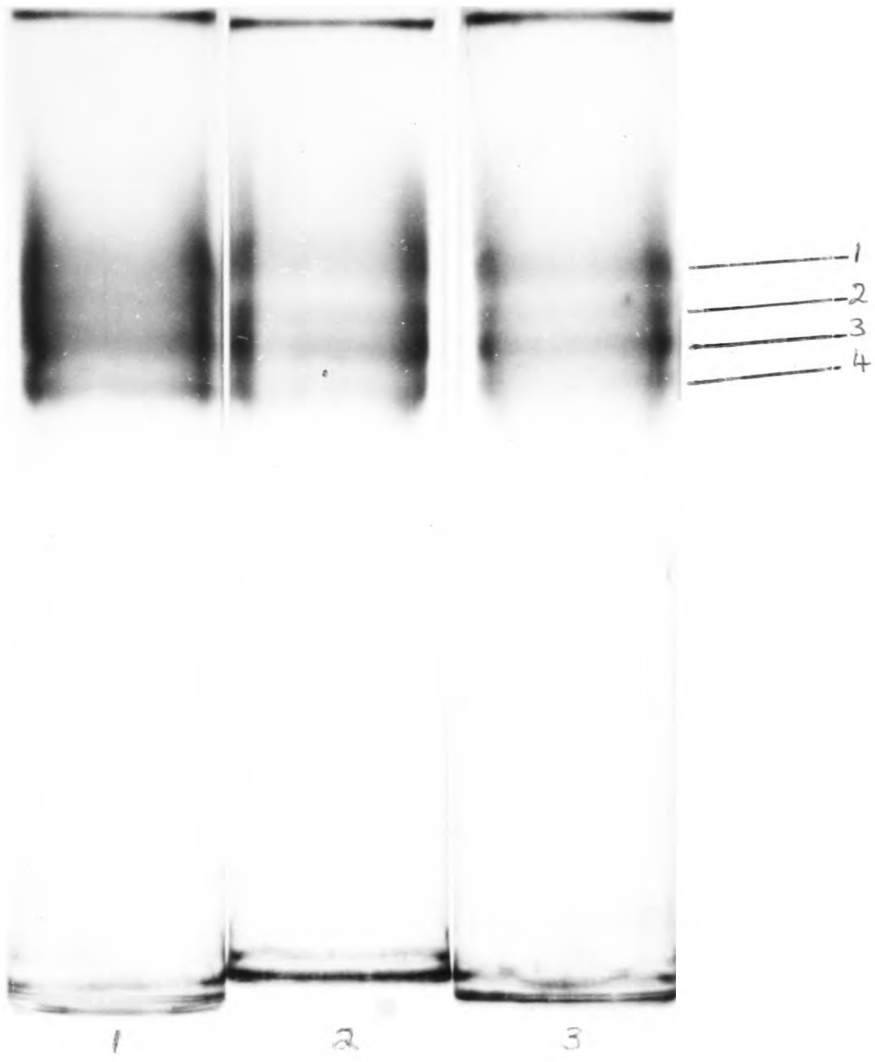


Figure 15 (b). The four bands of enzyme activity which appeared when the gels were stained for malic dehydrogenase.

with either NAD or NADP, they showed that of these three bands, two were of malic dehydrogenase (NAD) and the third band of malic dehydrogenase (NADP). In the present investigation, NAD only was used, so presumably all the bands are (NAD)-linked enzymes.

Protein solution which had been dialysed prior to electrophoresis showed a different pattern of enzyme activity. Segment 1 had only one band, and segments 2 and 3 had three bands all poorly defined. Dialysis had the same effect on enzyme bands as on protein bands, i.e. a general reduction in the definition.

Malic dehydrogenase from the soluble enzyme fraction from mitochondria showed the same pattern as the dialysed supernatant protein which was to be expected since the michondrial protein had also been dialysed. This effect of dialysis on the separation make it impossible to distinguish between the cytoplasmic enzyme and the mitochondrial enzyme which in other aspects had been found to differ (Davies, 1961).

Glutamic dehydrogenase. Fresh supernatant protein from the three segments of pea roots, when tested for glutamic dehydrogenase, produced 7 bands, (Figure 16a). The slowest moving of these bands was the strongest, and became broader during differentiation. The other 6, which were very fine sharp bands, decreased in intensity during differentiation.

After storage of the protein at -20°C for 3 days, the enzyme pattern showed another band of faster mobility than those previously obtained (Figure 16b). This band was strong in segments 1 and 2, but hardly visible in segment 3. Of the six bands which had appeared in the first separation, the fastest moving had increased in intensity considerably, in segment 1, but not in 2 or 3. The band of slow mobility was again much stronger in segment 3.

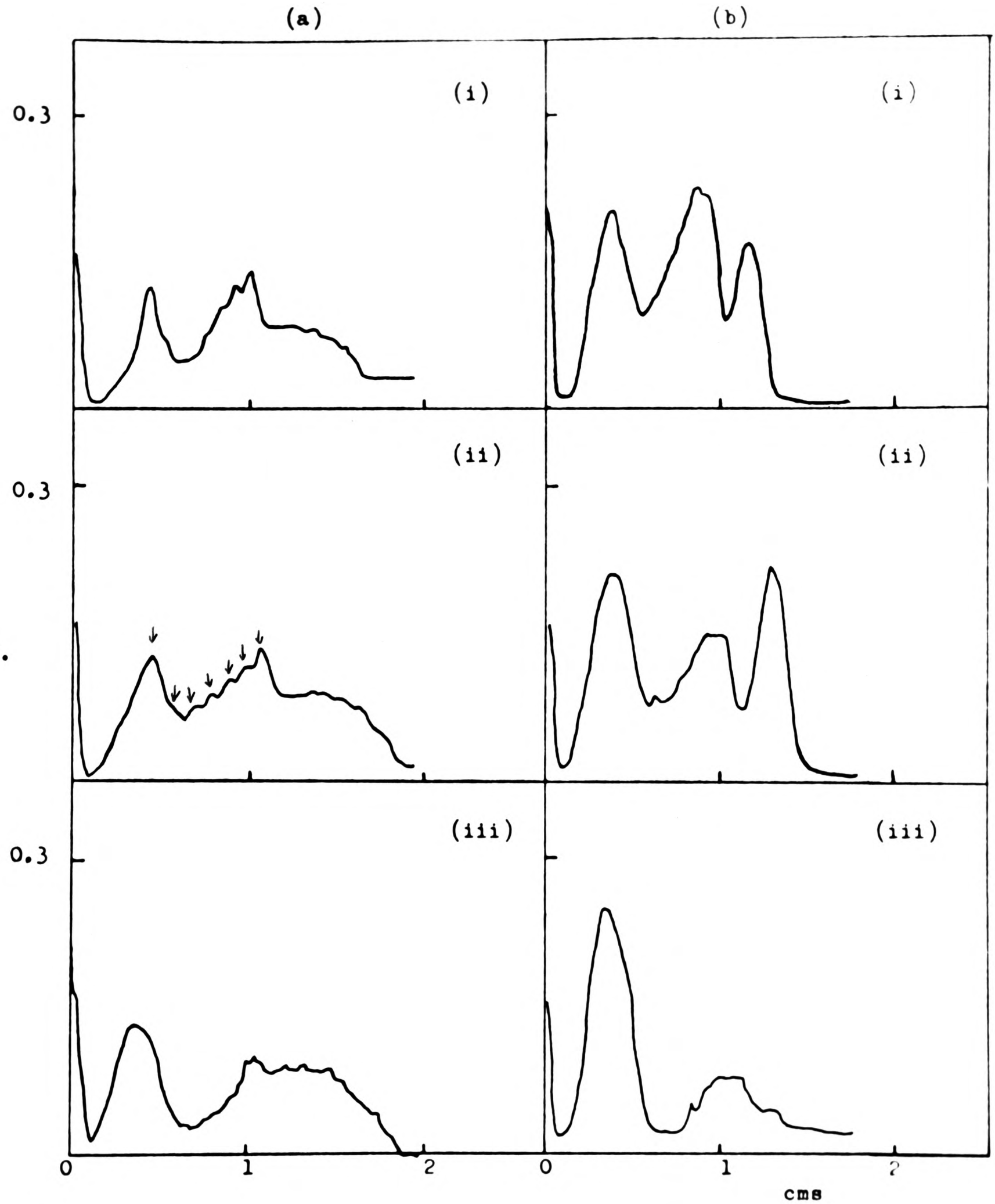


Figure 16. Glutamic dehydrogenase isozymes from the 3 segments of the pea root tip: (a) freshly prepared protein, (b) protein stored at -20°C for 3 days.

After further storage the band of highest mobility (which appeared after 3 days storage) had weakened.

Protein from mung bean mitochondria after storage had a similar pattern, to Figure 16b (segment 2), though the enzyme was much more active. After further storage the high mobility band had disappeared. (There are no results available from fresh protein.) It appeared that the same changes were taking place during storage, in the enzymes from both sources.

Succinic dehydrogenase. An enzyme test on fresh protein had a negative result, but after storage for 10 days at -20°C gels incubated with substrate, coenzyme and dye showed one band of enzyme activity. This was strongest from the meristem (Figure 17).

The changes in enzyme activity which occur during storage may be due to the activation or inactivation of enzymes or their inhibitors.

The results show that there are multiple forms of the same enzymes within the root and that the activities of the individual components are different in cells at different stages of differentiation. In other words, the relative activities of the isozymes change during differentiation.

When these enzyme studies were commenced it was hoped to correlate the activity of some enzymes with particular protein bands. The heterogeneity of the enzymes showed that such a correlation was impossible and the suggestion by Steward et. al. (1965) that the visible protein bands of pea roots represented particular enzymes, is probably not true. The appearance of many bands of activity of different enzymes in the same region of the gel, suggests that any protein band probably contains the activities of several enzymes. It is equally possible that the protein bands represent non-enzymic protein (Morris, 1966), and the coincidence of an enzyme band with a protein band, fortuitous.

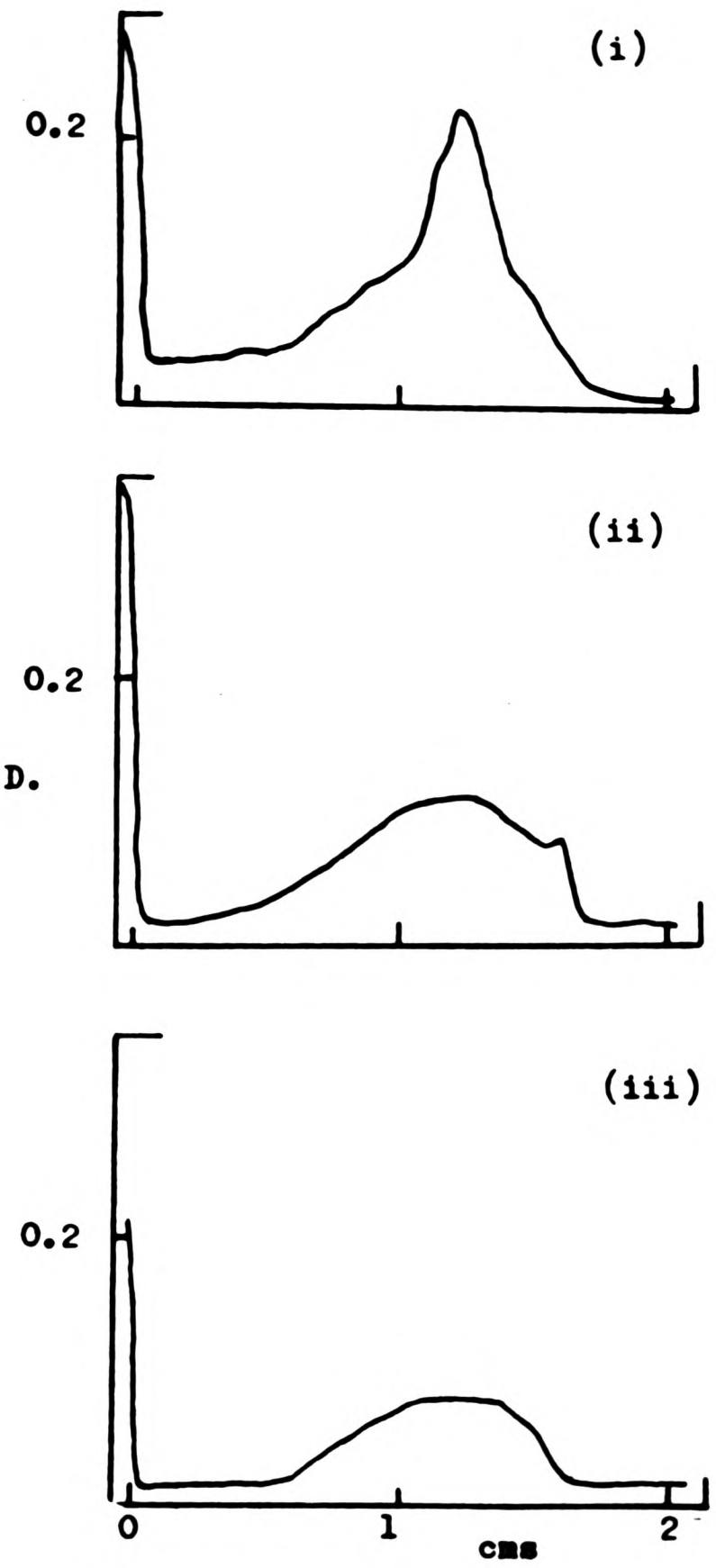


Figure 17.

Succinic dehydrogenase from segments of the pea root.

The heterogeneity of enzymes, i.e. the existence of multimolecular forms which differ from species to species and also from individual to individual, seems to be the rule rather than the exception, and the possibility also exists that non-enzymic proteins are equally heterogeneous. If this is the case, the appearance of distinct protein bands when such a mixture of proteins is fractionated becomes even more remarkable.

The postulate of "one gene, one enzyme" must be held in question because it is not known whether isozymes represent the products of one gene which have become modified during differentiation for specialized functions within the cell, or if they differ in primary structure and therefore represent the products of different genes.

V. INCORPORATION OF ^{14}C-LEUCINE INTO SUBCELLULAR PARTICLES OF PEA ROOTS.

(i) Introduction.

(a) As a preliminary to measuring the incorporation of labelled leucine into the soluble proteins, its incorporation into the particulate fractions of the cell was studied. It was hoped that results would show which particles were synthesizing protein and therefore which were responsible for producing the protein present in the supernatant.

(b) The site of protein synthesis.

Evidence obtained by Caspersson (1941) and Brachet (1942) for the involvement of RNA in protein synthesis, pointed to the microsomes being the sites of protein synthesis, and this was followed by direct evidence from Hultin (1950), Keller, Zamecnik and Loftfield (1954) and Borsook et. al. (1956). The microsome consists of membranous material and ribonucleoprotein particles (ribosomes) and the attribution of protein synthesis to the latter has come from many workers including Allfrey, Daly and Mirsky (1955).

The association of ribosomes in functional groups was postulated by Watson (1963) and Wettstein, Staehelin and Noll (1963), who compared the size of ribosome with the length of a molecule of messenger RNA. They came to the conclusion that one molecule of the messenger RNA was probably associated with several ribosomes. Electron micrographs of associations of ribosomes held together in clusters, which dissociated in ribonuclease, and evidence for their activity in protein synthesis (Warner, Knopf and Rich, 1963), support the conclusion.

Since these 'polysomes' vary in size it was thought possible that after the fractionation procedure used in this study, they would be found associated with more than cell fraction.

(c) Types of incubation.

Pea seedlings can either be given a long incubation (1 hour or more) in a labelled amino acid, sampled at intervals and the incorporation into protein with time measured, or they can be given a short 'pulse' incubation in labelled amino acid followed by a 'chase' incubation in the same amino acid unlabelled. Following a short pulse incubation with a chase incubation makes it possible to detect which cell fractions are retaining the protein they synthesize and which are releasing newly synthesized protein to other cell fractions.

In this study the pulse and chase method of incubation was used. To detect the cell fraction into which the amino acid is first incorporated the pulse should be so short that the newly synthesized protein does not have time to be transferred to another fraction. However, to give such a short pulse would be technically impossible. During the time that the amino acid takes to reach the protein synthesizing mechanism, the intercellular spaces become filled with the labelled amino acid, so a chase would necessarily be delayed. On the other hand, the pulse must be long enough for a detectable amount of the amino acid to get into the protein.

First experiments were designed to find a suitable pulse time.

(ii) Methods.

(a) Incubation procedure.

Pea seedlings were grown and harvested as described in section II (v). 60 seedlings with roots 3.5 - 4.0 cms were placed in a rack which was set on a dish of water. They were first given a pre-incubation of from 30 - 90 minutes to allow them to settle down to growth on a liquid medium. During this and subsequent incubations, which were carried out

at 25°C ± 2°C, the roots were aerated by occasionally lifting them out of the medium.

The period of preincubation is important. It has since been found (G. Bull, unpublished data) that the transfer of peas from vermiculite to water is a sufficient shock to cause polysomes to break down. It takes about an hour for them to recover.

At the end of the preincubation, the rack of seedlings was removed, the roots drained by touching them against the side of the dish, and placed in another dish containing 10 ml of pulse medium. This contained 0.5 μC/ml of generally labelled L-leucine-^{14}C of specific activity 160 mC/mM. Samples of this were removed for counting before and after the incubation. The pulse time for most experiments was 5 minutes.

After the pulse incubation, the seedlings were transferred to chase medium containing 100 μg/ml of non-radioactive L-leucine.

Samples of 10 roots were withdrawn at the end of the pulse, and at intervals during the chase. As they were withdrawn, the seedlings were immersed in ice cold water, and when they were cold the root tips excised. For some experiments the 7 mm tips were excised, for others the root tips were cut into the three segments using the perspex cutting device. First samples taken were kept at 0°C until all had been withdrawn. The root tips were homogenized and centrifuged by the method described in Section II (vi).

(b) Purification of the protein.

Protein from the particulate fractions obtained after differential centrifugation was purified by a modification of the method used by Mans and Novelli (1961).

The pellets were dissolved in cold 0.5 M NaOH (0.5 ml) and transferred to MSE conical tubes. A sample of the supernatant was also

treated to find the radioactivity of the soluble protein. In early experiements the dissolved pellets were transferred to pieces of Oxoid cellulose acetate membrane and the protein precipitated and washed on the membrane, but there were difficulties in digesting the protein off the membrane when this was required for measuring quantity of protein. This method was abandoned although it was technically much simpler.

The protein was precipitated by adding 0.5 ml of 10% trichloracetic acid (TCA) in the presence of added non-radioactive leucine (100 μg/ml). It was centrifuged and washed, by resuspending and centrifuging, in the following solutions:

TCA 5% cold

TCA 5% at 85^{o}C for 30 mins. (to hydrolyse and extract RNA and amino acid-charged RNA)

TCA 5% at room temperature

Alcohol/ether 50/50 at room temperature

Alcohol/ether at 37^{o}C for 30 minutes (to remove TCA and lipid material)

Ether.

The ether dry pellet was dissolved in N NaOH (0.2 ml) heated at 50^{o}C for 60 minutes. 20 l of this was removed for protein determination, the rest was dried onto 3 mm filter paper for counting. This was done in liquid scintillator in the Packard tricarb liquid scintillation spectrometer, at an efficiency of 45%. The scintillator was 5 gms PPO, and 0.3 gms POPOP per litre of AnalaR toluene.

Protein was determined by the method of Lowry et. al (1951), see below.

The soluble protein from the 130,000 x g supernatant from some experiments was used for electrophoresis and the radioactivity associated with the components measured by the procedure described in section VI.

(c) Protein determination.

Reagent A 2% Na_2CO_3 in 1 N NaOH

B 0.5% $CuSO_4.5H_2O$ in 1% Na or K tatrate

C alkaline copper solution ... 50 ml A + 1 ml B

D dilute Folin reagent ... Folin-Ciocalteu reagent 1:1 with H_2O.

Protein samples of from 0.2 ml to 20 l, containing 0.8 mg to 0.08 mg protein were used.

To the protein sample was added 1 ml of solution C. This was allowed to stand at room temperature for at least 10 minutes, then 0.1 ml Folin reagent D was added. This is allowed to stand for at least 30 minutes after which time the colour is measured in the Unicam spectrophotometer at a wavelength of 750 mμ.

Bovine serum albumin was used for the standard.

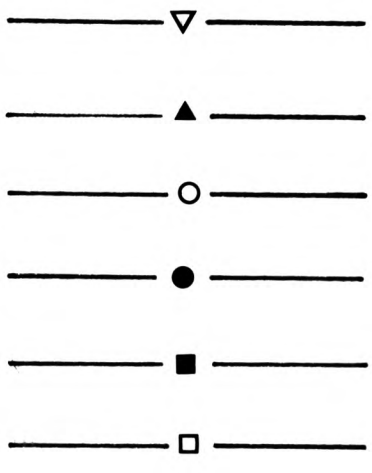
(iii) Results.

The specific activity of protein in the cell fractions from the 7 mm root tip after pulse time of 20 minutes, 10 minutes and 5 minutes were compared. The protein in all cases was sufficiently radioactive for counting, but only after a 5 minute pulse did any fractions lose activity when the seedlings were transferred to the non-radioactive chase medium. A pulse longer than 5 minutes obviously saturated the metabolic pools so that the labelled leucine was not significantly diluted during the chase incubation. A 5 minute pulse time was therefore chosen.

Results are shown in figures 18 - 21. Figures 18a and 18b show the total radioactivity and specific activity (the radioactivity per unit protein) of fractions from the 7 mm root tip, figures 19 - 21 show the specific activity of the three segments, 0 - 1.6 mm, 1.6 - 3.4 mm, and 3.4 - 6.4 mm described previously.

Figures 18 - 21. Incorporation of ^{14}C-leucine into protein purified from the subcellular fractions of the pea root. Pulse incubation 5 minutes in 0.5 µc/ml ^{14}C-leucine (160mC/mM), chase incubation in ^{12}C-leucine (100 µg/ml).

Extraction medium sucrose (0.5 M); tris (30 mM); HCl (24 mM); $MgAc_2$ (1 mM) KCl (50 mM): pH 7.6 at 0°C. Protein precipitated with TCA and washed by a modification of the method of Mans and Novelli (1961).



Mitochondrial fraction

fraction X

fraction V

ribosomal fraction

soluble protein

supernatant.

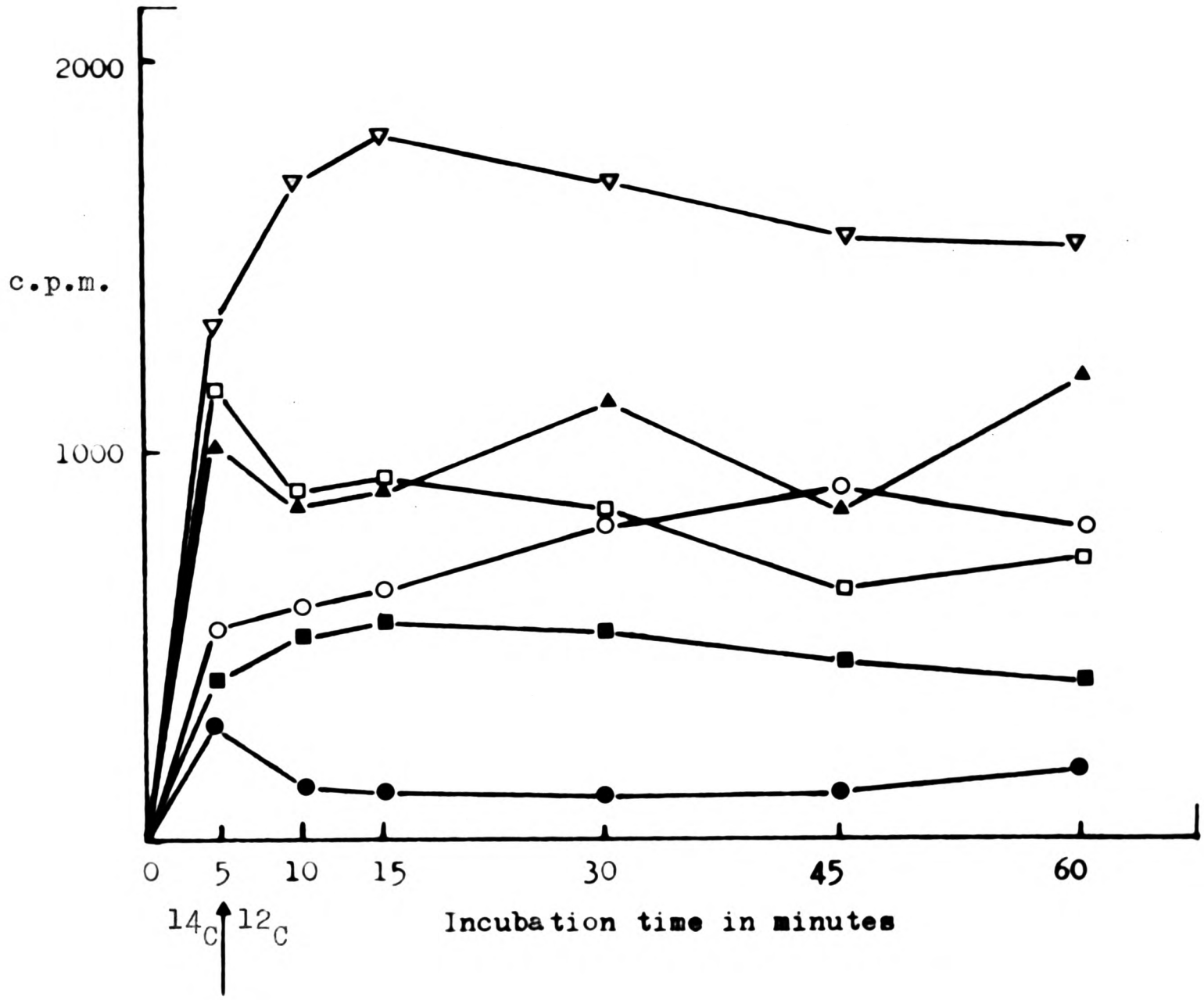


Figure 18 (...). Total radioactivity of fractions from the 7 mm root tip.

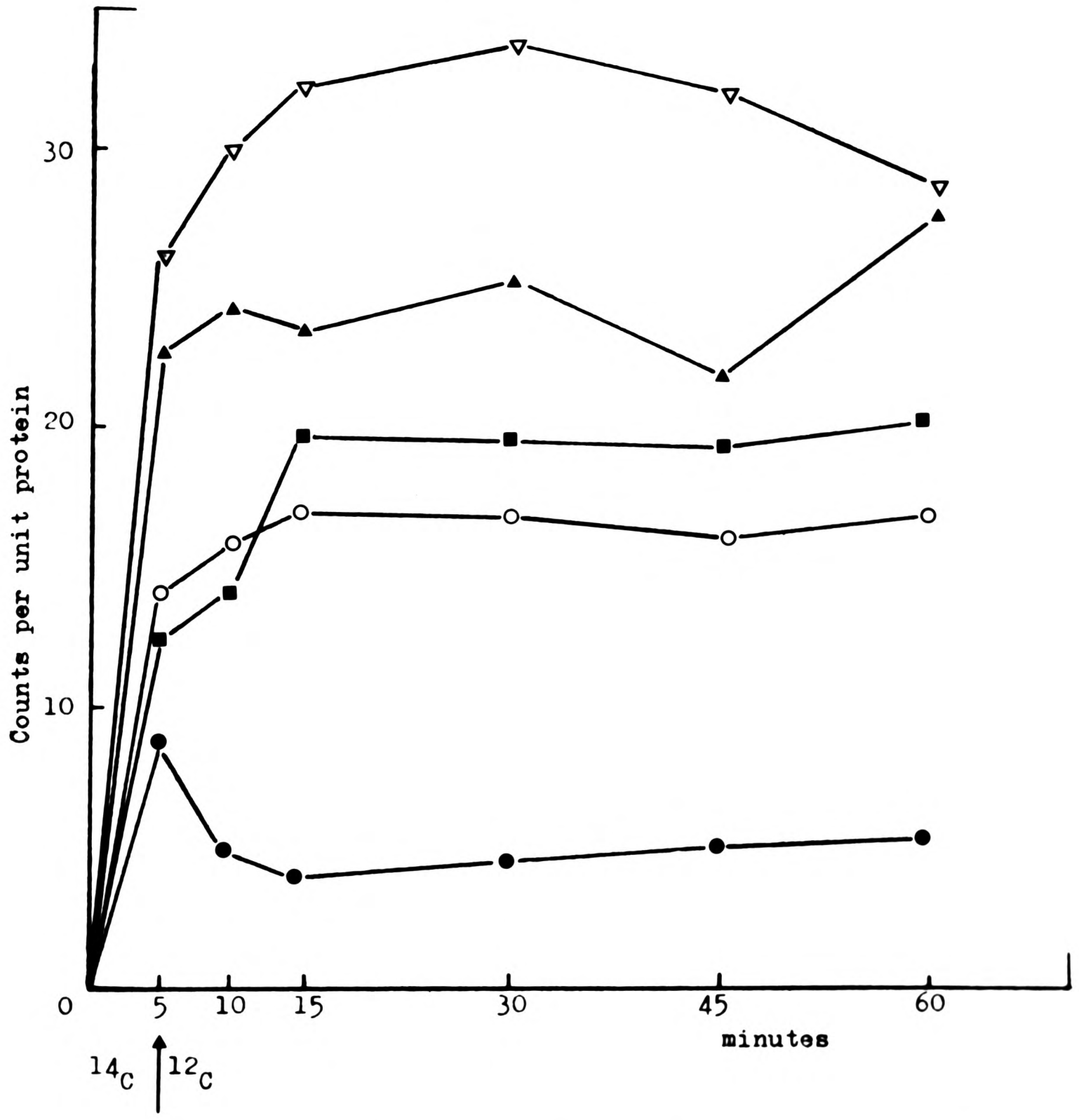


Figure 18 (b). Specific activity of fractions from the 7 mm root tip.

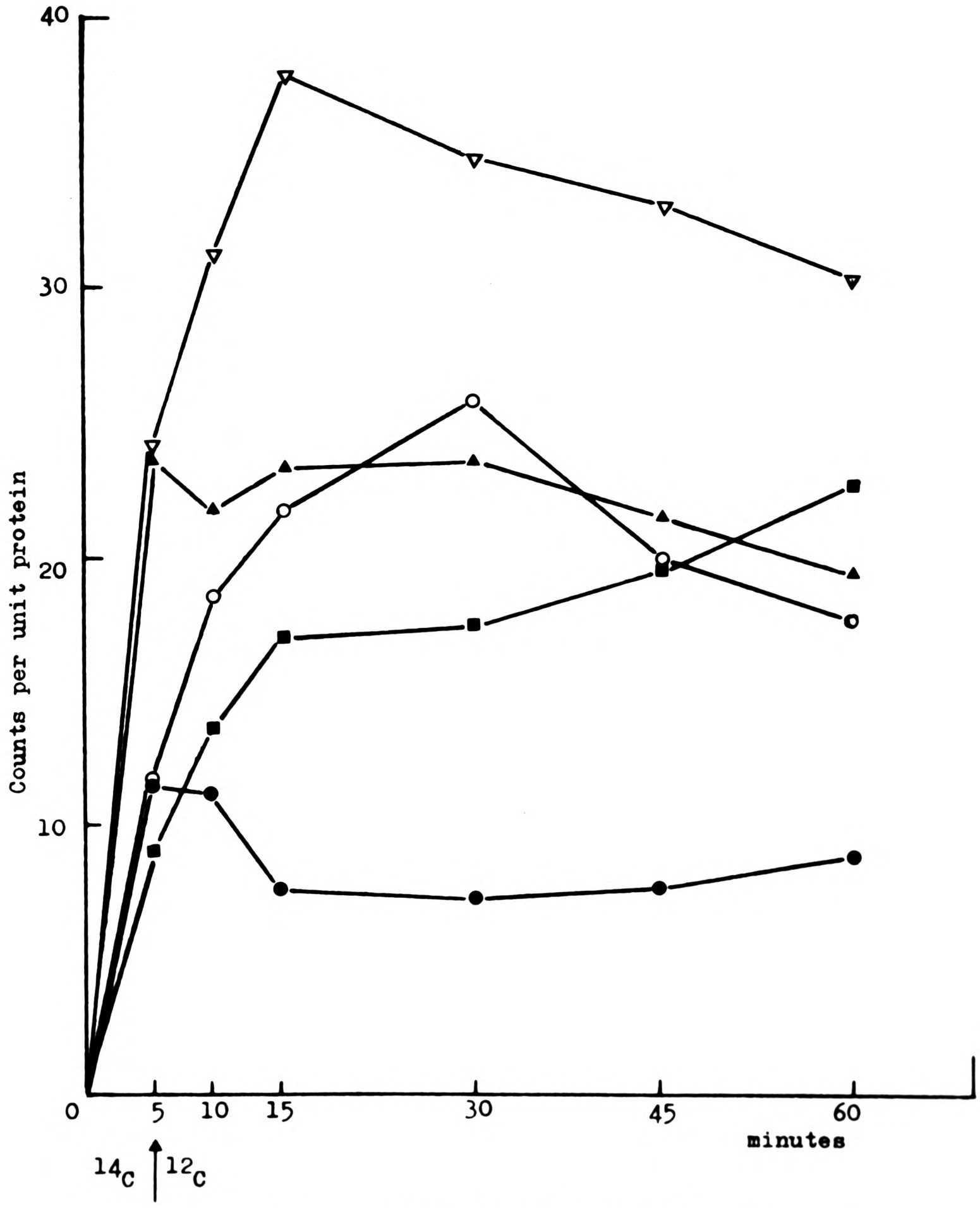


Figure 19. Specific activity of fractions from Segment 1.

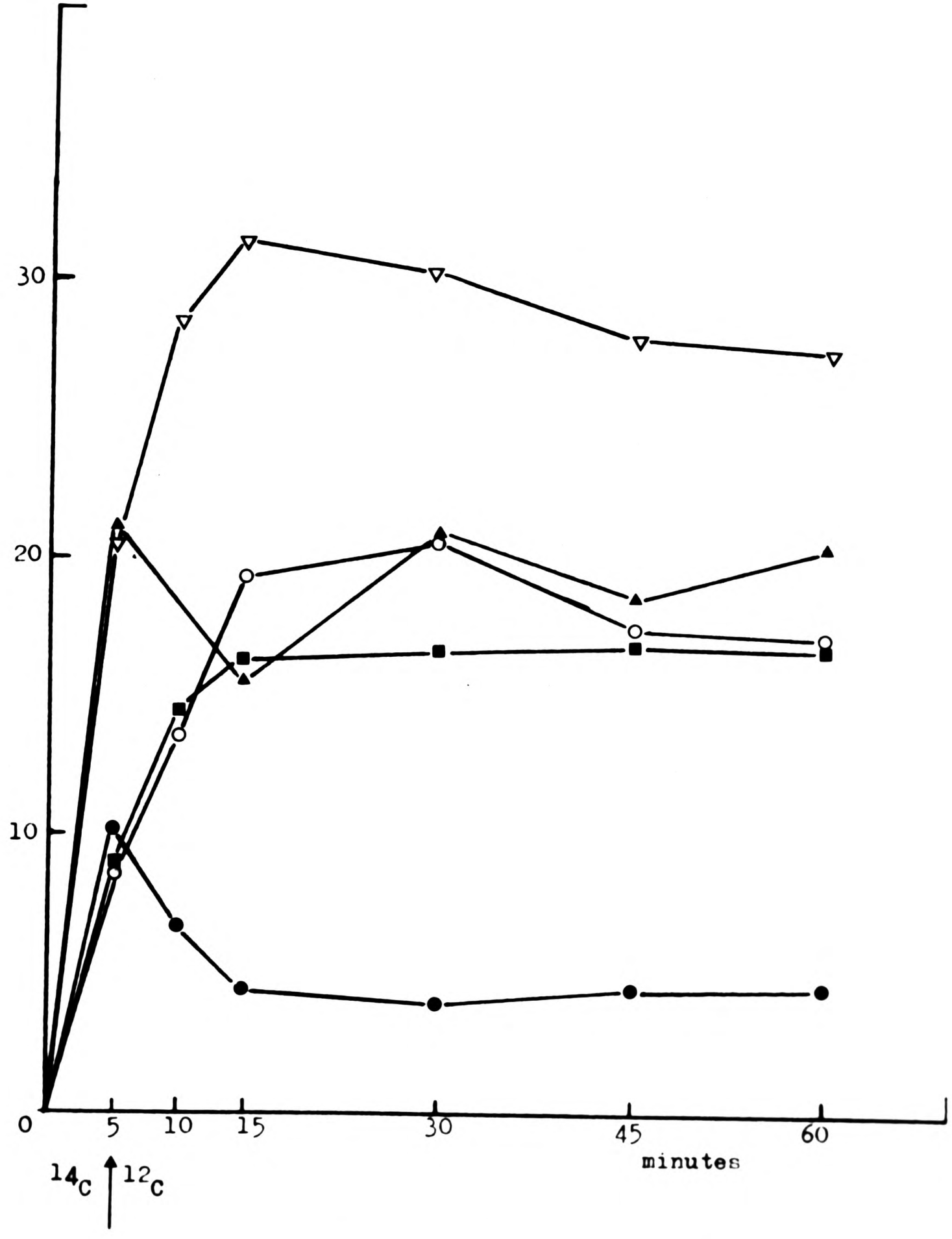


Figure 20. Specific activity of fractions from segment 2.

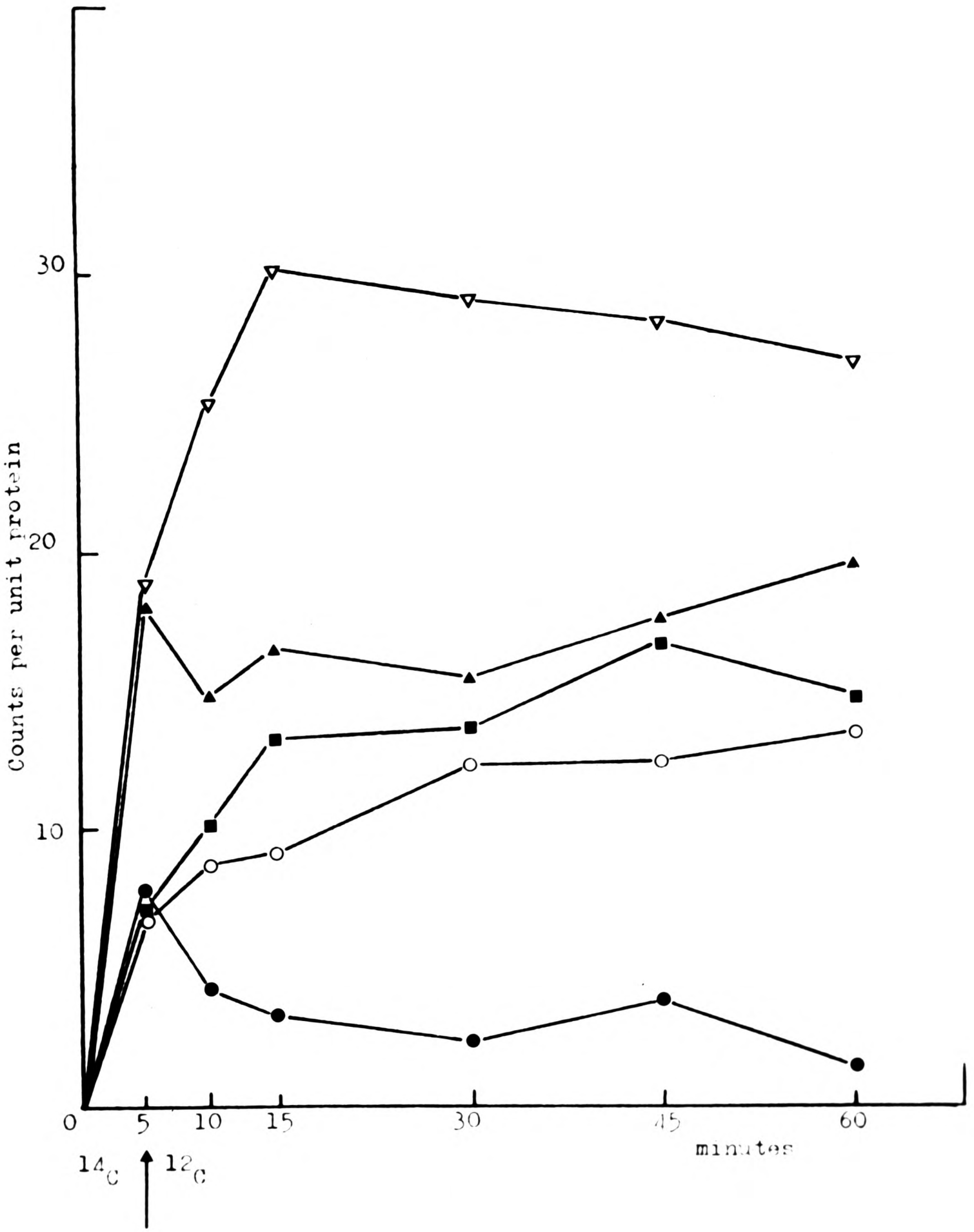


Figure 21. Specific activity of fractions from segment 3.

It must be stressed that the fractionation procedure used was crude and that no steps were taken to purify the particles from the fractions, e.g. the mitochondria from the mitochondrial fraction, so protein synthesis can only be ascribed to the fraction. The electron micrographs shown in Section II give some idea of the components of these fractions. No account has been taken of plastids, Golgi bodies of lysosomes, all of which were probably present in the intact cells. It is possible that plastids sediment with the mitochrondia, but that Golgi bodies and lysosomes disintegrate during the extraction procedure. The contamination of any fraction with even a small amount of a very radioactive cell component would lead to a false picture of the synthetic activity of the major component of the fraction.

It would be well to consider here the meaning of a rise or fall in the radioactivity associated with any fraction. There are three possible situations: a rise in radioactivity during the pulse incubation, a rise in radioactivity during the chase, and a fall in radioactivity during the chase. A rise in radioactivity of a fraction at any stage represents an accumulation of proteins, though this need not be due to protein synthesis by that fraction. It is not possible to distinguish between the site of origin and the destination of a protein.

A decrease in specific activity in any fraction represents a loss of protein from it, but this may be due either to a release of protein or to its breakdown.

The ribosomal fraction was the only one which consistently lost radioactivity (so that its specific radioactivity was decreased) when the seedlings were transferred to a non-radioactive medium: this was sufficient evidence that the chase was effective. It suggests that the route of the supplied leucine to the mechenism synthesizing protein in

the ribosomes was a direct one. Had it not been so, there would have been a lag between the transfer of seedlings to the non-radioactive medium, and the decrease in the radioactivity associated with the protein.

The immediate loss of activity from the ribosomes also suggests that the protein synthesised by them was very quickly transferred to some other cell fraction. However, they did not lose all their radioactivity. If the remaining activity (40%) represents an incorporation into structural ribosomal protein, it suggests that protein precursors are incorporated into ribosomal protein much faster than RNA precursors are incorporated into ribosomal RNA. Loening (1964) showed that RNA precursors appear in ribosomal RNA only after 50 minutes of incubation.

Another possibility exists that the proportion of radioactive protein associated with the ribosomes at any time during the chase reflects the proportion of labelled leucine still available for protein synthesis by the ribosomes in the cell at the time of sampling compared with amount available at the end of the pulse.

The results from the 3 segments showed differences in the labelling of ribosomes in cells at different stages of differentiation. The fact that ribosomes from the meristematic cells retain more radioactivity than those from expanding or mature cells probably reflects a synthesis of ribosomal protein, which is to be expected if, in dividing cells, all the components are being rapidly synthesized. The result may also be correlated with the degree of vacuolation in these cells. If the leucine eventually finds its way into the cell vacuole, and is only available to ribosomes while still in the cytoplasm, this would explain why in the meristematic cells where vacuoles are only just beginning to form, the leucine is available for longer.

The idea that leucine becomes unavailable to the mechanism which is synthesizing protein is supported by the measurement of protein and non-protein radioactivity in the supernatant. If all the free leucine in the cells is eventually incorporated into protein, the graphs of the amount of total label in the supernatant and the amount of label in the supernatant protein should approach each other and, at the point where the last of the freeleucine is incorporated into protein, they should meet. But the graphs show no sign of doing this (figure 18a). The difference between the amount of leucine in protein and the total amount of labelled leucine in the supernatant represents the amount of free leucine which has become unavailable for protein synthesis.

Because the radioactivity of the debris fraction, in most of these experiments was not measured, and because the amount of leucine removed during the purification of protein is not known, a complete balance sheet of labelled leucine in the cell cannot be drawn up.

The activity of ribosomes in protein synthesis is probably greater than these results suggest because any finished protein which has been released from the ribosomes will not be sedimented in this fraction. However, although the ribosomes in the ribosomal fraction may be responsible for all protein synthesis which occurs in these cells, the rapid rate at which the other cell fractions accumulate protein suggests that some of these are active in synthesizing protein. The immediate loss of activity from some other fractions when the seedlings are tranferred to a non-radioactive medium also argues in favour of this.

Protein in the other particulate fractions after 10 minutes usually has a specific activity much higher than that of the ribosomes and the generalization can be made that the heavier the particle, the faster its it synthesizing, or at least accumulating protein. In some experiments

the ribosomes, after 5 minutes did have a higher specific activity than either fraction V or the soluble protein, but they were rapidly overtaken by both of these fractions in the next 5 minutes.

The continued increase (during the chase) in activity by these other fractions may be due to the fact that they consist of larger and more complex structures than the ribosomes, and accumulate a lot of labelled amino acid during the pulse, which is only diluted slowly during the chase. The mechanism which synthesized protein within these fractions would thus continue to draw upon pools of labelled amino acid.

The fraction heavier than the ribosomes which appears to be releasing newly finished protein is fraction X, from which there is often a small loss of radioactivity immediately the seedlings are transferred to chase medium. This, together with some evidence from G. Bull (unpublished data) suggests that fraction X includes polysomes.

An interesting feature of the specific activity of fraction X is its decrease between 30 and 45 minutes, followed by an increase between 45 and 60 minutes. This 'dip' occurred in over half the experiments and always after the same length of time. It was therefore considered to be a genuine effect, and to represent a rhythm in the functioning in the components of this fraction. The increase in the radioactivity of this fraction between 45 and 60 minutes, suggests that it is tapping some pools of leucine which are not available to other cell components.

The appearance of the 'dip' only from segment 2, when the root was cut into the smaller segments, suggests that it is a feature of expanding cells, but an effect such as this, which probably varies slightly in time, could easily be missed if it occurred between samplings.

From a study of the electron micrographs, it appeared that fraction V, one of the components of the microsome fraction, was morphologically

similar to fraction X, but the specific activity of its protein during the pulse and chase incubations was different from either the ribosomes or fraction X. There is therefore no evidence that this fraction is synthesizing protein which is released to other fractions. The specific activity of V between 30 and 60 minutes of incubation varies slightly in the three segments: in the meristem it loses more radioactivity than it does in the older segments.

The protein of the mitochondrial fraction had a higher specific activity than that of any other fraction. It was slightly higher even than the debris in the experiment in which that fraction was analysed. The increase in radioactivity by the mitochrondrial fraction may be attributed either to the synthesis of protein by the mitochondria or by any non-mitochondrial material which sediments with them, or to the accumulation of protein synthesized in some other fraction and subsequently transferred to the mitochondria. However, there is so much evidence for mitochondrial protein synthesis (e.g. Das and Roy, 1961; Roodyn, Reis and Work, 1961; Parthier, 1963) that it is probable that some of the incorporation into this fraction is due to the synthesis of protein by the mitochondria themselves. Mitochondria probably contain a complete mechanism for synthesizing protein. There is evidence that they contain RNA (Reis, Coote and Work, 1959), that they contain ribonucleoprotein particles (Rendi, 1959) which appear to have the same biosynthetic activity as those in the microsome fraction, and that they contain DNA (Luck and Reich, 1964; Woodward and Munkres, 1966).

The rate of incorporation of leucine into the mitochondrial fraction was rapid over the first 15 minutes, then fell off very quickly, possibly when all the labelled leucine which may have accumulated inside the mitochondria during the pulse incubation was used up.

VI. INCORPORATION OF ^{14}C-LEUCINE INTO THE SOLUBLE PROTEINS OF PEA ROOTS.

(i) Methods: Sectioning and dissolving polyacrylamide gels.

When the incorporation of labelled leucine into the particulate fractions of the three segments had been measured, the labelled soluble proteins were fractionated on polyacrylamide gels and the radioactivity associated with each of the bands measured. It was hoped that this would show which protein bands were being synthesised in the different regions. If the new band is a result of de novo synthesis of proteins, it should have a higher specific activity than the other proteins.

Following electrophoresis of radioactive protein, the polyacrylamide gels were cut into sections so that the radioactivity in each section could be measured. The gels were sectioned using a device based on a wire egg slicer. (A similar cutter has been described by Heinman, 1964). It consists of two pieces, one of which had a hemicylindrical trough in which the gel was placed. This had slots in it at 1 mm intervals. The second piece had wires stretched taught on a frame which fitted over the first piece so that the wires went into the slots.

To facilitate cutting, gels were first frozen in dry ice for 15 - 20 minutes. This removed the springy elastic property of the gel so that accurate 1 mm sections could be cut. The wires were pressed slowly through the frozen gel. The sections were removed with a forceps and placed in Packard bottles.

The gel sections were dissolved in 30% hydrogen peroxide, a method discovered by Young and Fulhorst (1964), and reported earlier in a short communication to Canalco Disc Electrophoresis Information Centre. It was necessary to dissolve the sections, to increase the efficiency of counting the radioactivity. Because of the self absorption, it would not have been

possible to get a high enough proportion of the counts by measuring the disc directly.

Hydrogen peroxide, 0.4 ml, was added to each bottle. These were loosely capped and placed in an oven at 50°C for several hours. The time taken for the gels to dissolve was 5 hours or longer. The dye with which the gels had been stained was decolourized by this treatment. A piece of 3 mm filter paper, 2 cms square was placed in each bottle to absorb the hydrogen peroxide, and the filter papers were then dried by replacing the bottles, uncapped into the oven at 50°C for 20 - 30 minutes.

When the paper was dry, 6 ml of toluene scintillator (5 g PPO, 0.12 g POPOP per litre of Analar toluene) were added to each bottle, and the radioactivity counted in the Packard tricarb liquid scintillation spectrometer.

There was a risk of losing the -COOH group due to oxidation during the hydrogen peroxide treatment, but since the leucine was generally labelled, no more than 1/6 of the total should have been lost that way.

(ii) Results.

The results of a 5 minute pulse are shown in Figure 22.

In the meristem, the radioactivity scan follows the optical density scan very closely. This suggests that in the meristem all proteins are being synthesized at approximately equal rates. This is to be expected since cells which are undergoing division will be synthesizing all cell components and all their proteins. The scan of radioactivity associated with the soluble protein from the expanding zone is different.

The appearance of the new protein band suggested that a new protein was being synthesized, but it was also possible that it had been formed by the rearrangement of existing molecules. The scan of radioactivity associated with the soluble protein from the expanding zone showed a

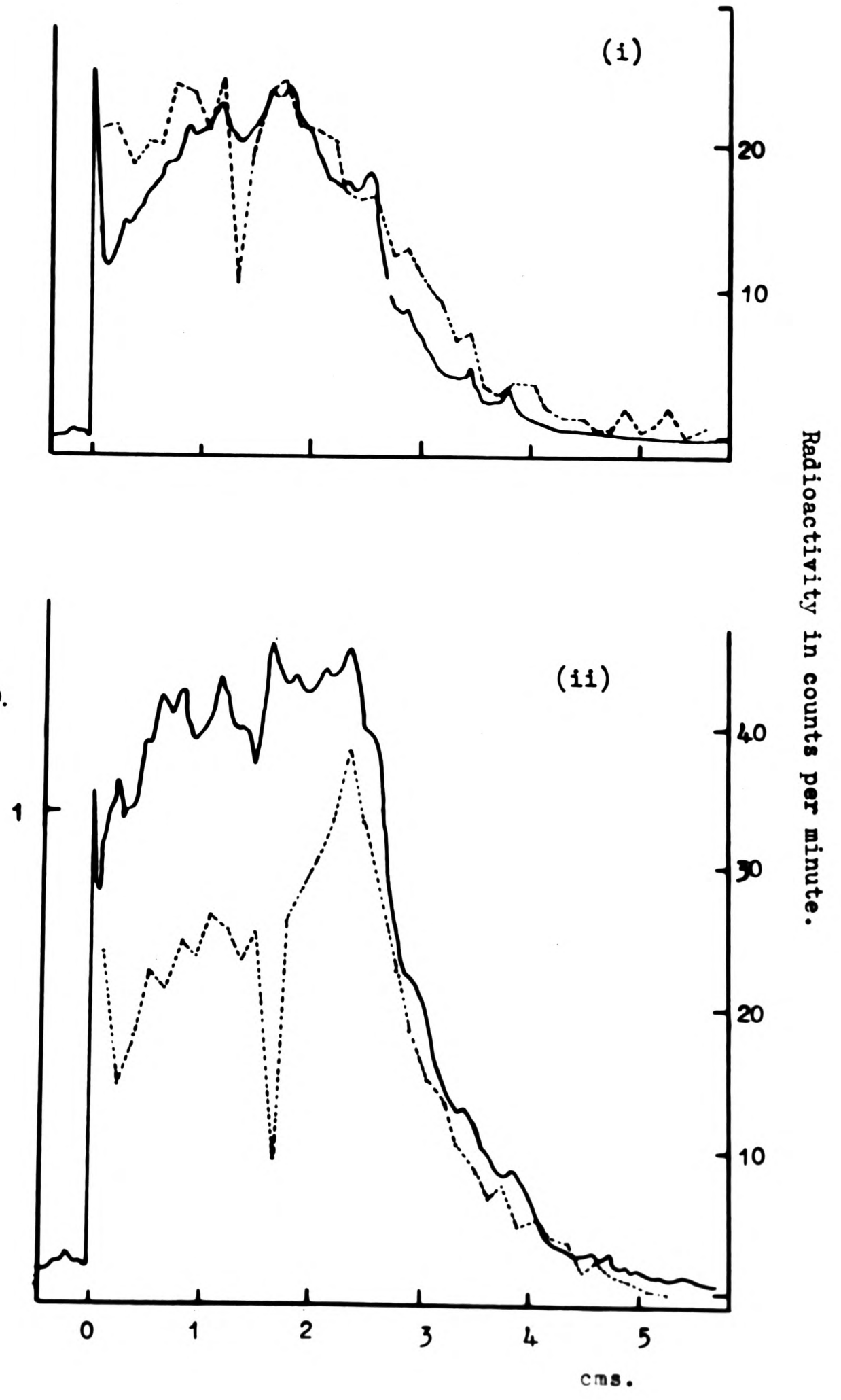


Figure 22. The incorporation of labelled leucine into the soluble proteins of the pea root. (i) Segment 1, (ii) Segment 2.

———— O.D., - - - - - - - - Radioactivity.

large peak of activity in the region of the new band which showed that the protein of this band was being rapidly synthesized. It had previously been considered possible that the new band had been formed by the rearrangement of molecules already present in the meristematic cell, but this result shows that it represents the de novo synthesis by expanding cells of a protein which is not synthesized by meritematic cells.

This protein was also being synthesized in segment 3, but not quite so rapidly as in segment 2. The radioactivity associated with the other bands from segments 2 and 3 did not follow the protein scan closely. This showed the rates of synthesis of the various soluble proteins also change during differentiation.

(The overall activity of the soluble proteins in segment 2 was lower, because the uptake of leucine into this region was lower than it had been into segment 1. Also there is a lag between the end of the pulse and the appearance of maximum radioactivity in the soluble fraction.)

Figure 23 shows segment 2 after 5 and 10 minutes in chase medium. After 5 minute chase there is still a lot of radioactivity associated with the new band but after a further 5 minutes, most of this had disappeared. This suggests that the protein of the new band is being broken down. It must therefore be turning over rapidly.

There is evidence too that protein in other regions of the gel undergoes turnover. If all the soluble proteins were stable there would be no change in the radioactivity of the bands once they had reached their maximum activity. Results from the three segments showed that during the 55 minutes chase incubation there was much variation in the general pattern of radioactivity in the bands.

Results of a 10 minute pulse incubation followed by a 20 minute chase incubation, followed the same pattern as those from the

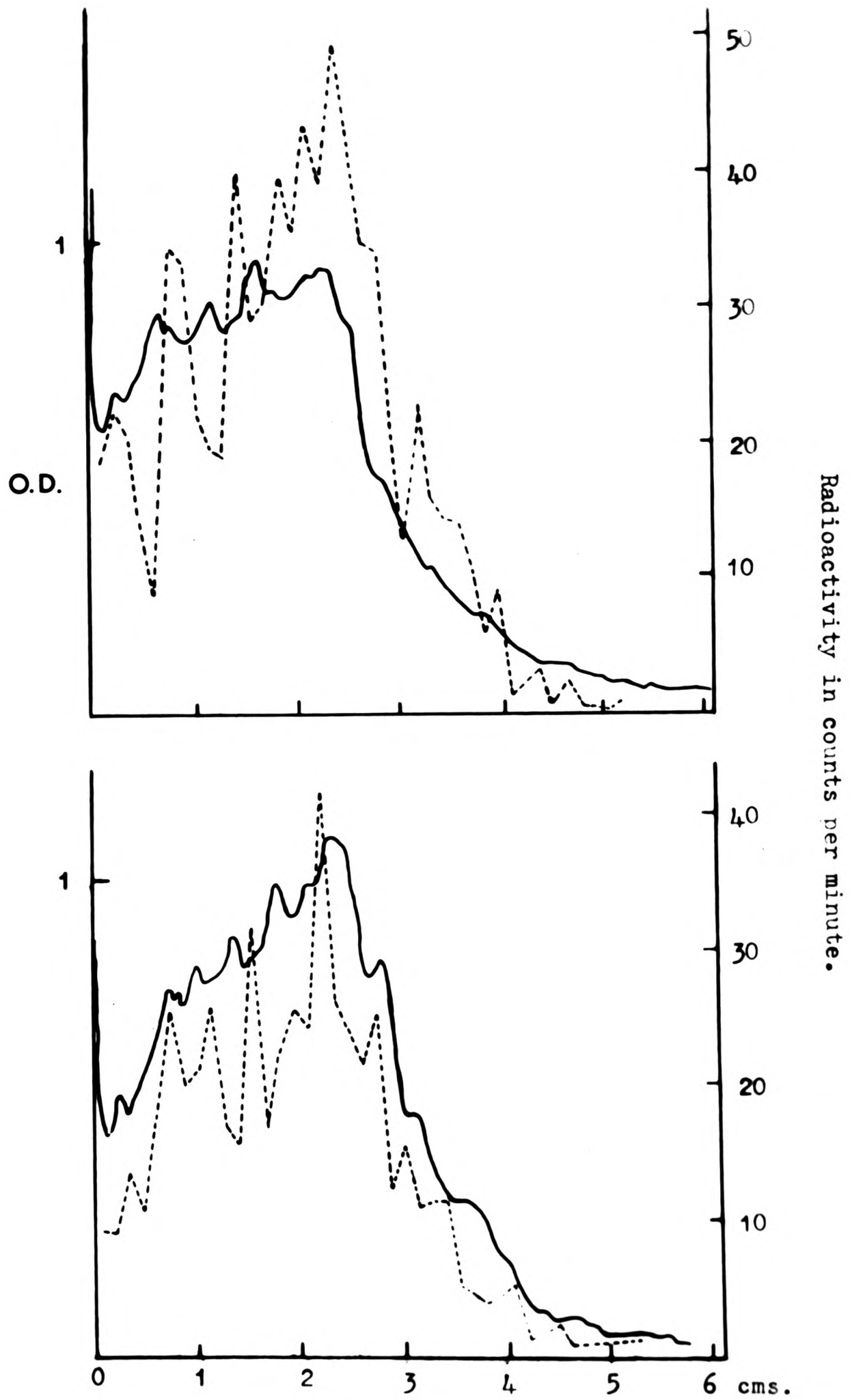


Figure 23. The loss of radioactivity from the new band during the chase incubation: (a) 5 min. pulse and 5 min. chase, (b) 5 min. pulse and 10 min. chase.

———— O.D., --------- radioactivity.

particulate fractions after the same length of pulse, i.e. there were no visible effects of the chase incubation. This too suggests that over 5 minutes in labelled precursors is long enough to saturate the metabolic pools.

It is of little value to discuss the specific activities of the protein bands because it is not known how many protein components are contributing to the radioactivity of any band.

An important point must now be considered which has not yet been resolved. If the total radioactivity on each gel is calculated and compared with the radioactivity of protein precipitated from an equivalent volume of supernatant, a discrepancy is found.

The difference may represent a loss of ^{14}C during the hydrogen peroxide treatment of gel slices, or it may represent a change in some protein molecules so that they become either too large to enter the gel (e.g. if they are converted to some structural protein) or so small that they pass right through the gel, or that they become positively charged and migrate to the cathode.. The discrepancy increases with time, which suggests a gradual change in the protein molecules.

VII. INCORPORATION OF ^{14}C-LEUCINE INTO THE SUBCELLULAR PARTICLES OF CULTURED ROOT SEGMENTS

(i) Methods.

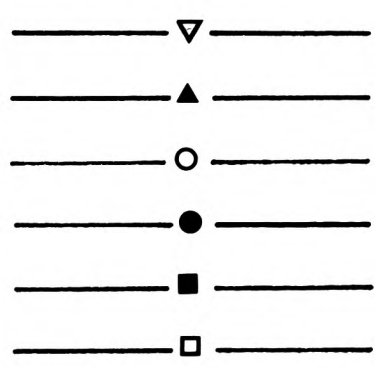
For these experiments 7 mm root tips or the expanding segment (1.6 - 3.4 mm) were excised and grown in a 2% sucrose medium, in the dark, on a rotary shaker rotating at 80 - 120 rev./min. At the end of the culture period they were transferred to perforated tubes, washed and these placed in larger tubes each of which contained ½ ml of pulse medium. After 5 minutes they were drained, washed and transferred to chase medium (pulse and chase media as described in section V (ii)). As they were removed from the chase medium the root tips were cooled and tranferred to homogenizing tubes. The rest of the procedure was as described in section II (vi).

(ii) Results.

After a culture time of 5 hours the graphs of radioactivity associated with the subcellular fractions were very similar to the controls (fresh roots). Both fraction X and the ribosomal fraction lost radioactivity when the root segments were transferred to the chase medium, the loss from fraction X being slightly delayed. The one difference between these segments and the controls was the chase from the mitochondrial fraction. This fraction lost 20% of its radioactivity between 10 and 15 minutes of incubation, but increased again in the next 15 minutes.

If root tips were cultured for 16 hours, there was a very different pattern of incorporation (Figure 24). None of the particulate fractions lost any radioactivity during the chase. In fact they continued to increase in specific activity throughout the incubation.

Figure 24. 7 mm root tips excised and cultured for 16 hrs. in 2% sucrose. The graphs in this figure, and in Figure 18 (a), represent the total radioactivity in the mitochondrial fraction, fraction X, V and R, but only 1/20 of the total radioactivity of the soluble protein and the supernatant.



mitochondrial fraction
fraction X
fraction V
ribosomal fraction
soluble protein
supernatant

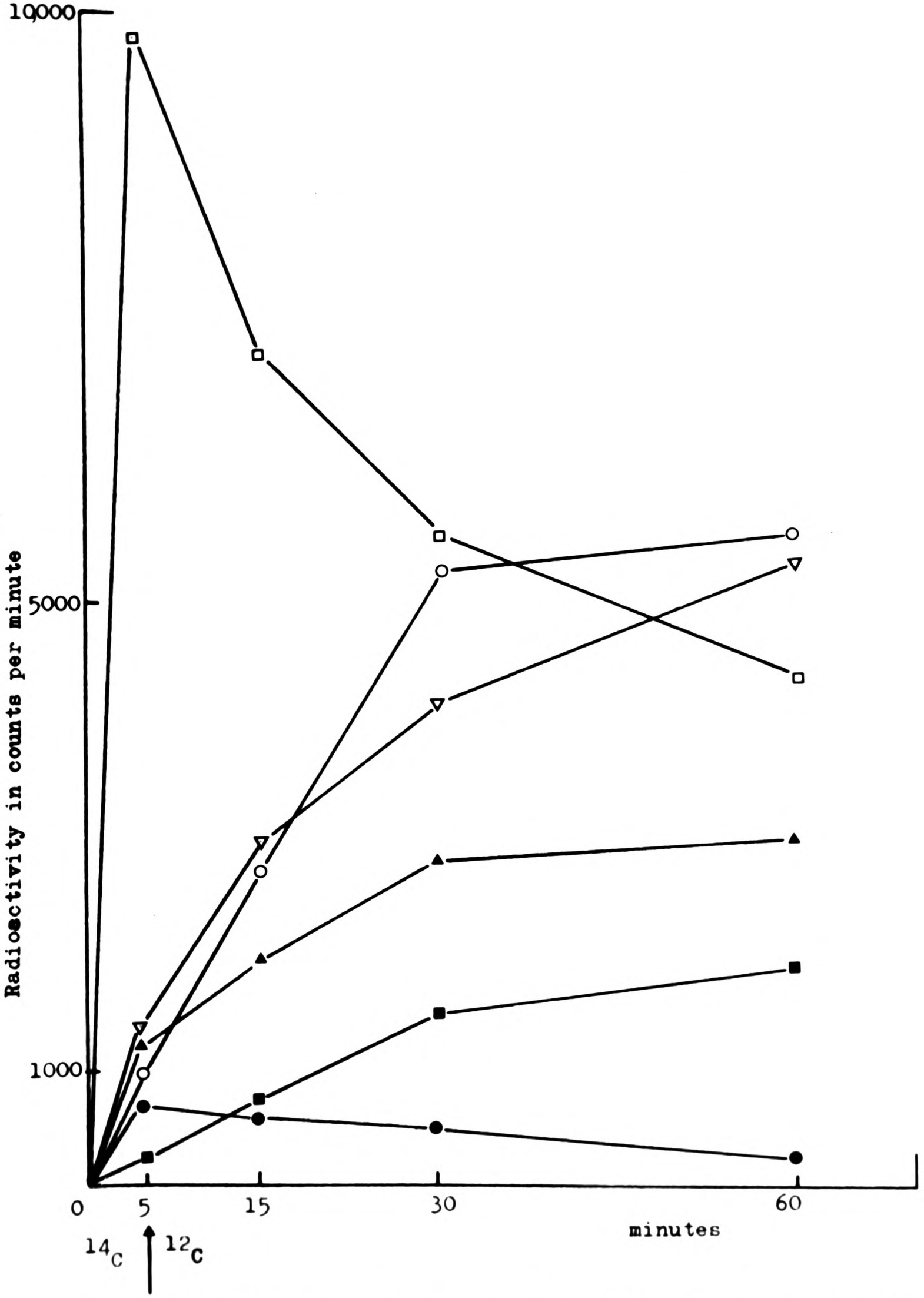


Figure 24. Incorporation of labelled leucine into the particulate fractions of excised roots.

This was due to the fact that these root tips absorbed considerably more labelled leucine during the pulse incubation, and the particulate fractions continued to draw upon this leucine during the chase. It seems that culturing root tips had the same effect on the uptake mechanisms as ageing does on discs of storage tissue, in which a prolonged washing results in the development of mechanisms which can absorb ions rapidly. This effect will be considered in the discussion.

In section III (vi) the effects of culturing on the components of the soluble protein were described. It had been found that during 16 hours of culture there was an increase in the amount of one of the slower moving bands. No evidence from these incorporations could be gained, for the correlation of the synthesis of this protein with any particular subcellular component.

The culture conditions used in these experiments were non-sterile so the possibility has to be considered that the leucine is being incorporated into bacterial protein. However, it was considered earlier (III (vi)) that bacteria should have been preserved intact by the extraction medium, and should have sedimented with the debris, or possibly the mitochondrial fraction.

An experiment in which the expanding segment (1.6 - 3.4 mm) was cultured for 16 hours and then given pulse and chase incubations showed a different pattern of incorporation from the 7 mm tip. It is therefore suggested that results described here are of pea root rather than of bacterial protein.

The results of the expanding segment showed that it, too, had developed a greater capacity to absorb leucine, but whereas fractions from the 7 mm tip continued to increase in specific activity during the incubations, those of the expanding segment decreased. The rate of decrease was rapid to 30 minutes, then slowed down considerably.

The radioactivity of the soluble protein increased slowly throughout the incubation. This was the only experiment in which fraction V lost a considerable amount of radioactivity (50%) immediately the segments were transferred to non-radioactive medium.

Such a loss of activity would be more characteristic of a fraction which contained polysomes. One can only speculate as to the changes in function which take place in the subcellular fractions of small segments under these conditions.

VIII. THE EFFECT OF ACTINOMYCIN D ON THE INCORPORATION OF ^{14}C-LEUCINE INTO PROTEIN.

(i) Introduction.

Protein synthesis is carried out under the direction of messenger RNA (mRNA) which carries the genetic information from the DNA to the ribosomes. Actinomycin D has been used to try to determine if all synthesis in pea roots is dependent on a continuous supply of unstable messenger RNA.

For the synthesis of a protein to stop, the life of the messenger, carrying the information which codes for this protein, must be of limited duration, i.e. unstable. Brown (1963) has postulated that differentiation is brought about by a series of messengers released by the nucleus during the course of development. These must have a limited life. By inhibiting the synthesis of RNA, i.e. the synthesis of further messengers, any protein whose synthesis is directed by an unstable messenger should stop when the remaining messenger has broken down. Pea seedlings were incubated in actinomycin D prior to pulse and chase incubations to find out how much protein was being synthesised in the absence of a continued supply of m RNA. Actinomycin D inhibits the synthesis of DNA-dependent RNA (Hurwitz et. al. 1962) by binding to the DNA template (Shatkin, 1962; Reich, Goldberg and Rabinowitz, 1962; Brachet and Denis, 1963) and forming a reversible complex with it (Kirk, 1960). It does not inhibit the synthesis of cellular DNA or of viral RNA (RNA-primed RNA synthesis) at concentrations inhibiting mRNA formation (Reich, Franklin, Shatkin and Tatum, 1961). In Bacillus subtilis, mRNA turnover and protein synthesis were completely inhibited (Acs, Reich, and Valansu, 1963; Levinthal, Keynan and Higa, 1962) which suggests that in this organism all protein synthesis depends on an unstable mRNA.

The effect of actinomycin D on tissues depends on the concentration in which it is supplied. Perry (1963) found that at 10^{-6}M all RNA synthesis was inhibited, but at 10^{-7}M there was still RNA synthesis in the chromatin. He also found that at a certain concentration (Perry, 1962) DNA dependent RNA synthesis continues, but that nucleolar RNA synthesis is stopped.

Levinthal, Keynan and Higa, (1962) used actinomycin D to establish the half life of the messenger, but it has been pointed out by Trakatellis, Axelrod and Montjar (1964) that the synthesis of mRNA may be linked to its breakdown, and preventing its synthesis may result in the measurement of an abnormally long duration time.

The synthesis of proteins in the presence of actinomycin D may be due to the messenger being stable but it has been shown by Paul and Struthers (1963) that some animal (fibroblast) cells can synthesise RNA in the presence of actinomycin D. It was not shown if this resistant RNA was DNA primed. The suggestion was made that it is a ribosomal RNA formed by an independent process, resistant to actinomycin D.

It has been stated that the effect which actinomycin D has on a tissue depends on the concentration in which it is supplied. It also depends on how easily it penetrates the cels. In case where permeability is low, the effective concentration (i.e. the concentration of the antibiotic inside the cells) is much smaller than the supplied concentration. If a cell is permeable to actinomycin D then 1 μg/ml is enough to prevent RNA synthesis.

Plant cells require a higher supplied concentration for effectiveness than animal cells. This is partly due to the presence of the plant cell wall, which, I have evidence to suggest, binds the antibiotic. It is known that if actinomycin D is supplied to pea roots at a concentration of 5 μg/ml, RNA synthesis stops (Loening, unpublished), but it has not

been shown how much penetrates further than the outer two cell layers. A transverse section of a pea root, grown into actinomycin D, showed the outer two cell layers to be deep yellow and the rest of the section very pale yellow. It appeared that the antibiotic was bound by the cell wall.

(ii) Results.

Both the 7 mm root tip and segment 2 (the 1.6 - 3.4 mm, expanding segment) have been cultured in the presence of actinomycin D (5 μg/ml). Sucrose (2%) was also present in the culture medium.

In the 7 mm root tip, cultured for 16 hours, the radioactivity incorporated into the cell particles was very similar to the controls (7 mm tips cultured for the same length of time in 2% sucrose), but the amount of radioactivity in all fractions was depressed (Figure 25).

The incorporation of leucine into the supernatant was also depressed (by 20%) and it was considered that the smaller amount of radioactivity in the particulate fractions was a direct result of the depressed uptake. However the inhibition of incorporation into the particulate fractions was greater than that into the supernatant. (It ranged from 40 - 70%.) These results suggested that there was also an inhibition of the mechanism of protein synthesis in the cells.

The percentage inhibition decreased with time which suggested that the tissue was beginning to recover from the actinomycin treatment (the antibiotic had not been present during the pulse and chase incubations.) There is evidence (Bal and Gross, 1963) that roots of Allium cepa can recover from the antibiotic, but only "catch up" with controls after a period of 3 days.

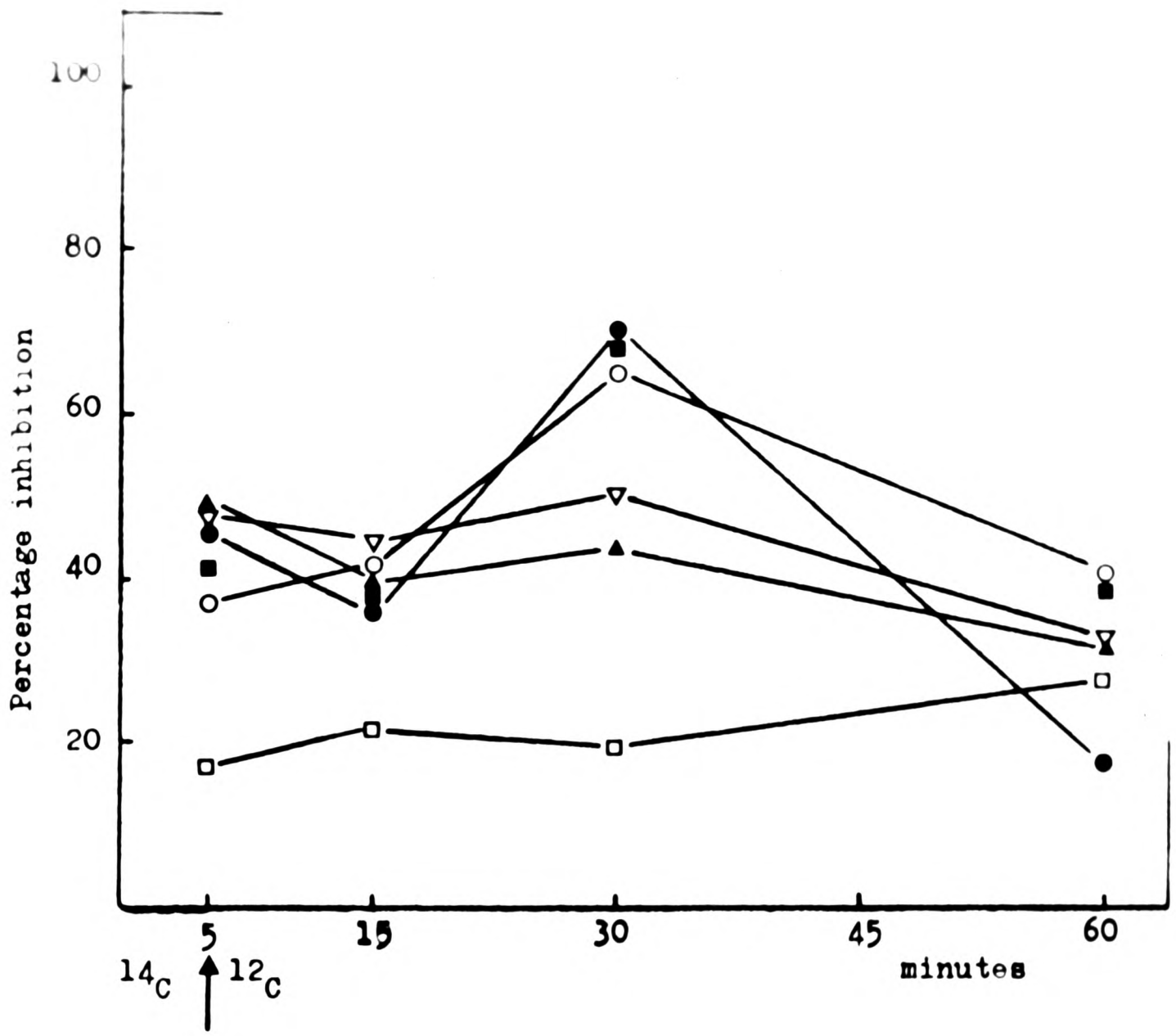


Figure 25. The percentage by which incorporation of radioactivity into protein of excised segments is inhibited by actinomycin D

A more probable explanation for the apparent recovery is that because the fractions are incorporating leucine more slowly, it remains available for longer.

To explain the decreased capacity of treated segments to take up leucine, it is not necessary to postulate its effect on a metabolic process concerned with ion uptake. If the growth of root tips is inhibited, it is quite possible that the treated root tips have volumes which are 20% less than the controls, and the uptake of leucine could be directly correlated with its volume. On the other hand the results suggest that actinomycin is interfering with metabolism and a depression of any metabolic process is likely to result, eventually, in a decreased capacity for ion uptake.

In the experiment on the expanding segments, actinomycin only depressed uptake of leucine by 5% but incorporation into the particulate fractions was depressed by 15 - 20%. The inhibition was only seen after 30 - 60 minutes because the chase from some fractions (X and V) was delayed.

The significance of these results will be discussed.

IX. DISCUSSION.

(i) Soluble proteins.

Electrophoresis on polyacrylamide gel provides an easy, if somewhat crude fractionation of plant proteins. By this method all the soluble proteins can be quickly separated on 3 - 4 cms of gel. Fortunately there are some groups of ions of the same mobility which are sufficiently large to stain up as protein bands. The number of bands is small compared with the number of proteins present in the cell, the rest of the proteins are present in amounts too small to form a band. Because there must be thousands of proteins represented by the background staining, very many changes could occur and not be detected by this method. An indication that the minor components are changing is found in the fact that the background staining decreases in intensity during differentiation. Fortunately some changes involved the major components of the protein pattern. The discovery of an intense new protein band from expanding cells was gratifying. Both Steward et. al. (1965) and Morris (1966) studying the changes during differentiation of the pea seedling root find a new protein which is of similar mobility.

Changes in relative intensities of bands might be correlated with a change in the relative volumes of different tissues present in the root. (There is a large increase in volume of the cortex compared with the stele.) It has been shown that the RNA contents of cells in the different tissues varies (Bucknall and Sutcliffe, 1965) and it is probable that the protein content of these cells is also different. A change in the proportion of tissues could not however be responsible for a new band. The appearance of the new band could not be correlated with any new morphological feature in the second segment, although there is a large increase in the size of the vacuole.

That a new band appears, implies the rapid synthesis of the protein: this is correlated with the relatively large amount of labelled leucine which is incorporated by the band. The conspicuous loss of radioactivity from this protein during the chase suggests either that the protein is being incorporated into the particulate fraction, or that it is characterized by a high rate of turnover. Cells which are expanding rapidly may be synthesizing considerable quantities of structural protein for which this new protein might be a precursor, but the appearance of the same band in the soluble protein from the mature cells of the most basal parts of the root argues against this. A protein with a high rate of turnover, through which the cell could bring about rapid changes in its metabolism may mediate differentiation.

The fact that there are quantitative changes in the protein of a cell during differentiation poses the problem of what controls the rate at which the various proteins are being synthesized or broken down, and how is this control effected. If differentiation is brought about by a changing balance of proteins its control must lie at the level where the rate at which particular proteins are being synthesized is determined. Since the evidence points to all somatic cells having the same genetical constitution, i.e. the same DNA, and since cells differ biochemically, either the relationship between DNA and the RNA template for protein synthesis cannot be direct or all the RNA templates are formed but some are not functional. It is, therefore, necessary to postulate some intermediate which restricts either the formation or the function of RNA. It has been suggested that chromosomal proteins (the histones) play some role in this regulatory mechanism.

There is evidence that the association of histone with DNA prevents its functioning as a template for RNA synthesis (Huang and

Bonner, 1962; Bonner, Huang and Gilden, 1963) so the attachment or detachment of histones could regulate the activity of the genes. Differentiation could be brought about in this way, and the control of differentiation would then lie in the factors which determine the behaviour of histone. Stedman and Stedman (1950) find that different cells within the same organism contain different histones, but the presence of different histones in cells suggests that they are also the end products of differentiation.

The currently favoured view, put forward by Jacob and Monod (1961) on the basis of results from experiments with bacteria, is that cells contain, besides genes which determine the structure of a protein, genes which determine whether or not that protein is being synthesized. In this system, the structural gene is switched by the operator gene which itself is repressed by a metabolite synthesized under the control of a regulator gene. This repression is only lifted when another metabolite (the inducer) combines with that produced by the regulator gene. The operator, no longer inhibited, initiates the activity of the structural gene. It is possible that a system analogous to that in bacteria is operating in higher organisms.

Some evidence for the switching of genes was obtained in the present results. The synthesis of protein by expanding cells which was not synthesized by meristematic cells implies that some genes are switched on during differentiation. A system where this is more likely to happen is in the transition from the dormant state in discs of storage tissue, to a state of intense metabolic activity during dedifferentiation in the developing callus. It had been shown (Robertson, 1966) that during the early stages of development of the artichoke callus, the number of protein bands which appear increases dramatically.

Some evidence that genes may be switched off is provided by the general decrease in the background staining (which represents the minor protein components) during differentiation. This is also supported by the work of Morgan and Reith (1954) who found that some material which reacted with ninhydrin was present in meristematic, but not in older cells.

However, that differentiation involves mainly quantitative changes in cell proteins, is supported by the constancy of the protein bands (other than the new band) from cells at all stages of differentiation. These proteins varied only in relative intensity of staining, and in the rates of incorporation of labelled leucine. The redistribution of radioactivity in these proteins during the chase incubation suggests that, like the new band, some of these too are turning over.

It was hoped that enzyme studies would help to identify some of the protein bands but when a gel was stained for a single enzyme, several bands of colour were formed. None of the protein bands could, therefore, be identified with the activity of one particular enzyme. The several bands of activity (isozymes) were greater in number and closer together than the protein bands in the same region of the gel. It was, therefore, considered that the coincidence of any protein band with a band of enzyme activity was fortuitous.

Results are consistent with the view that most enzymes are heterogeneous (Wroblewski, 1961). Studies of enzyme changes during differentiation until now have consisted mainly of measurements of the total amount of activity, and have not taken into account this heterogeneity. Although a measure of the changes in the total amounts of different enzymes is instructive, equally important are the changes which occur in the relative quantities of separable fractions of the same enzyme. The presence of

isozymes and the relative changes which take place between them form another facet of enzyme changes during development.

The difference in the mobility of the isozymes is due to a difference in their structure resulting in a difference in their net charge, or in their shape or size. A difference in primary structure would suggest that they were the products of different genes; a difference in folding only, would suggest that they may have been produced by the same gene and have been modified later by a cytoplasmic mechanism which must have emerged early in differentiation. Whatever their origin, the fact that their relative activity is changing suggests that they are under separate control.

The biological activity of enzymes may be controlled by specific metabolites which bind to the protein causing a conformational alteration or allosteric transition (Monod, Changeux and Jacob, 1963). Such an alteration could result in enzymes being converted from an inactive form to an active form. Evidence for this has come from Sundaram and Fincham (1964) who found a mutant enzyme in Neurospora crassa which was inter-convertible between electrophoretically distinct active and inactive forms. In the present study, it was only possible to detect changes, during development, in the relative intensities of the enzyme components. Such changes have also been found by McCune (1961) and by Cahn, Kaplan, Levine and Zwilling (1962).

The numbers of isozymes and their mobilities vary from one species to another, from organ to organ and, as these results show, from one stage of development to another. It has also been shown that different isozymes exist in different subcellular fractions (Ting, Sherman and Dugger, 1966). These workers found that in young maize roots, mitochrondria contained 2 isozymes (of malic dehydrogenase) of slower mobility than the

1 cytoplasmic form. In pea roots, 2 mitochrondrial isozymes were detected which had slightly faster mobility than either of the 4 cytoplasmic forms. This illustrates the variability of isozyme forms. If each form represents the most suitable configuration for a specific function, then the cell's synthetic processes are extremely versatile.

(ii) Particulate fractions.

Labelled leucine was incorporated into all subcellular fractions but the specific activity of the protein in each was different. In the pea root, the ribosomes had the lowest specific activity, the mitochondrial fraction and the debris fraction had the highest. A similar result has been found in pea epicotyl (Ts'o and Sato, 1959), in the bean hypocotyl (Webster, 1964), in carrot explants (Sutcliffe, Bollard and Steward, 1960), in tobacco leaf discs (Stevenson, Thimann and Zamecnik, 1956) and in yeast (Cooper, Harris and Neal, 1962).

The distribution of mechanisms for protein synthesis throughout the cell suggests that it is more efficient for the cell to synthesize protein at the site where it is needed than to transport large protein molecules from one part of the cell to another. There is much evidence for protein synthesis by particular organelles, e.g. nuclei (Allfrey, Hopkins, Frenster and Mirsky, 1960) and mitochondria (Das and Roy, 1961; Parthier, 1963; Haldar, Freeman and Work, 1966).

The results of the synthesis of mitochondrial protein obtained by Haldar et. al. are interesting. They studied the rate at which whole mitochondrial protein becomes labelled and the rate at which one particular soluble protein (cytochrome c) from mitochrondria becomes labelled. They found that the rate at which cytochrome c is labelled parallels closely the rate at which cytoplasmic proteins are labelled and

is different from that of whole mitochondrial protein. This suggests that at least some proteins are synthesized at one site and transported to another.

Evidence for the existence of ribonucleoprotein particles with similar properties to ribosomes in most cell organelles (Ts'o, 1962) suggests that the mechanism by which proteins are synthesized is the same in all cell fractions. The activity of the ribosomes in different organelles may differ. The small number of ribosomes in the mitochondrial fraction compared with the ribosomal fraction, suggests that if leucine incorporated into this fraction is due to protein synthesis carried out by mitochondrial ribosomes, they must be exceedingly active.

A change in the number of mitochondrial ribosomes during development of animal cells has been shown by Andre and Marinozzi (1965), who found that mitochondrial ribosomes are more numerous in tissues of embryonic Rat, than in tissues of adult Rat. This suggested that mature mitochondria were less active in protein synthesis. The 12% decrease in the specific activity of the mitochondrial protein during development found in the present experiments could not be attributed to a reduction in protein synthesis by this fraction: it probably reflected the slightly lower uptake into the supernatant, by older than by the meristematic regions of the root. This larger amount of leucine in the supernatant of the meristem may be the result of either a greater absorption by this region or a transport towards the meristem of leucine taken up by the older regions.

Leucine is chosen for incorporation experiments because it is a relatively unreactive amino acid. The radioactivity in the proteins has been ascribed to leucine but the specific activity of this amino acid in the proteins has not been measured and the possibility exists that

during the hour of incubation, some has been converted into another product. The finding that at the end of the incubation a considerable amount of radioactivity is still in non-protein form in the supernatant suggests that it is unavailable for protein synthesis. It has been considered that labelled leucine may become unavailable for protein synthesis due to its being secreted into the vacuole, but the disposal of such a useful molecule into the vacuole must be questioned. It is more probable that the radioactivity is associated with some other compound.

It has long been considered difficult to measure protein breakdown because of the probable re-use of the breakdown products for further protein synthesis. However, it has recently been shown (Steward and Bidwell, 1966) that the amino acids resulting from the breakdown of proteins are preferentially used for respiration.

The presence of proteolytic enzymes in the cells of growing roots suggests that protein breakdown is a feature even of actively growing root cells. Present results which demonstrate protein turnover support this. It is, therefore, possible that labelled leucine released when proteins break down is converted to some other compound and in this way the radioactivity associated with its carbon is lost to protein synthesis.

(iii) Cultured segments.

The growth and metabolism of root segments in culture depends on the length of the excised segment (Brown & Wightman, 1952), the region from which it is cut (Robinson and Brown, 1954) and the medium in which it is cultured (Brown and Sutcliffe, 1950; Vaughan, 1965). That excision does not inhibit gene activity was shown by Loening (1965), who found that although excised segments do not synthesize ribosomal RNA (during 2 hours of culture), they continue to synthesize an RNA which has the properties of a messenger. Studies on other aspects of the metabolism of cultured root segments

have also shown that metabolism of these segments is different from that in the intact root.

The difference between the protein bands obtained from the soluble proteins of excised and intact root segments reflects the different metabolism in the two situations. The new band is again present, and after 24 hours in culture it increases in intensity relative to the other bands. The proteins in one other region of the gel also increase during culture. This does not compare with the increases in enzyme activity which reach a maximum after 6 - 12 hours (Robinson and Brown, 1954 and Vaughan, 1965).

Labelled leucine was again incorporated into all subcellular fractions and no difference could be detected in the relative activities of the fractions compared with those in the intact root. The main difference between the two was the continued incorporation of radioactivity into protein in the particulate fractions of the excised roots. This was considered to be the result of a much greater uptake of leucine into the supernatant during the pulse incubation.

The reason for the increased uptake was not considered to be the increase in surface area. (This was shown by a control experiment.) It must, therefore, have been due either to the transfer of segments from a medium containing sucrose to one which did not, and in which they would swell slightly, or to a change in the inherent capacity of the cells for leucine incorporation.

Discs of tissue cut from a storage organ immediately increase in metabolic activity and develop the capacity for ion absorption (MacDonald, Knight and DeKock, 1961). It is postulated that inhibition of these processes within the organ is effected by an inhibitor which is volatile in nature. This postulate is based on the fact that excised discs kept in air develop this response.

The situation with roots differs, in that the pieces of tissue (root segments) have been cut, not from dormant, but from an actively growing plant organ. However, excision seems to have similar effect: the absorption is increased. This could be attributed to a leakage of the inhibitor through the cut surface. Evidence for the presence of the inhibitor in growing roots comes from Laties and Budd (1964). They found that by stripping the cortex off the stele, the stele could be switched to a metabolism which resulted in ion uptake.

In the present experiments the effect of actinomycin D on cultured root segments was to depress the uptake of leucine by 20%. This could be attributed either to an inhibition of the ageing process, or to the smaller volume of the treated root segments, whose growth had been inhibited by the antibiotic. The difficulty of using antibiotics to investigate metabolism, is to decide what constitutes a control. If a root tip grows from 7 to 8 mms in the presence of actinomycin and from 7 to 12 mms in the absence of the antibiotic, it may be more instructive to compare the treated root tip not with the whole of the control, but with the apical 8 mms.

However, since the capacity of a tissue for the absorption of any substrate depends on its metabolism, it is likely that inhibition of any metabolic process will lead to an inhibition of uptake. The fact that the inhibition of protein synthesis by cell fractions was greater than the depression of uptake, suggested that the inhibition was not entirely due to the lower concentration of leucine inside the cells. Some other mechanism of inhibition was also operating.

The effect of actinomycin D was a slight overall depression of synthesis. It was not a dramatic effect, and there was no evidence

of preferential inhibition of protein in any particular fraction. The components of the soluble fraction following electrophoresis were not affected.

These results suggested that in pea root cells, protein synthesis is not entirely dependent on a continuous supply of unstable messenger RNA. Other systems have also been found in which actinomycin has little effect, e.g. Spiegel, Ozaki and Tyler, (1965), found the same pattern of protein bands and radioactivity in sea urchin embryos fertilized and grown in the presence of 20 µg/ml actinomycin D. Paul and Struthers (1963) working with L.S. fibroblast cells suggested that an actinomycin resistant RNA synthesis primed either by RNA or DNA may be taking place.

Although protein synthesis continues in root cells treated with actinomycin D, differentiation is inhibited (Bal and Gross, 1963). From this it would seem that differentiation is brought about by some proteins whose synthesis depends on a continuous supply of an unstable template and which are, therefore, preferentially inhibited by actinomycin D. It was suggested earlier that the new protein band consists of an unstable protein through which the cell might control differentiation. However, the appearance of the new band in root segments treated with actinomycin D does not support this.

Compared with a fractionation procedure such as that involving precipitation with ammonium sulphate the technique of polyacrylamide gel electrophoresis is very simple. It is, however, a technique by which only 15 or so of the major components, out of the thousands of different proteins present in a cell can be fractioned, but this proved to be a sufficiently sensitive method for detecting at least some of the changes which take place in the proteins of pea root cells during differentiation.

Combining the fractionation of proteins on polyacrylamide gel with tests for enzyme activity on the separated proteins, greatly increases the sensitivity of the method, and by using this technique further changes during differentiation involving enzyme proteins have been demonstrated.

Methods have recently been devised for dissolving structural proteins, so it should now be possible to extend the method of polyacrylamide gel electrophoresis to a study of the changes, which take place during differentiation, in proteins associated with the particulate fractions of the cell.

X. SUMMARY.

1. Soluble proteins from plant tissue can be successfully separated on polyacrylamide gel into several well defined protein bands. From the soluble fraction of pea roots, fifteen or more bands are obtained.
2. The effect on the protein pattern of different methods of gel polymerization, various buffer systems, and different extraction procedures has been studied.
3. Proteins from different parts of the pea seedling produce different patterns of protein bands.
4. Proteins from cells at different stages of differentiation within the root produce different patterns. Scans of these show the net changes which take place in proteins during differentiation.
5. The main feature of these changes is the appearance of a strong protein band from expanding and fully expanded cells which was not present in the meristematic cells. There are also changes in the relative intensities of protein bands which appear from all stages of differentiation, i.e. other changes which take place during differentiation are quantitative rather than qualitative.
6. The new band is more rapidly labelled than the other proteins during a short incubation in labelled protein precursor which suggests that it is more rapidly synthesised. There are also differences in the rates of synthesis of the other protein bands.
7. Enzyme proteins are separated by electrophoresis into several components (isozymes). Their relative intensities change during differentiation suggesting that either they are the products of different genes, or they are located at different sites in the cell and are modified by their local environments.

8. The protein pattern obtained from excised segments changes during a 24 hour culture period. The changes are different from those which occur during differentiation. There is a relative increase in the intensity of bands in two regions of the gel.
9. These changes do not take place in 1 mm segments or in the 1.6 mm apical segment: in these the protein bands become indistinct.
10. A labelled protein precursor (leucine) is taken up by the pea seedlings and incorporated into the protein of all cell fractions studied. The rates of accumulation of radioactive protein by each fraction differ. The mitochondrial fraction has the highest and the ribosomal fraction the lowest specific activity.
11. There is a transfer of radioactive protein from the particulate to the soluble fraction, but transfer of radioactivity from one particulate fraction could not be detected.
12. When seedlings are transferred from a radioactive to a non-radioactive medium, the activity in the superantant decreases, but of the particulate fractions only the ribosomes, and occasionally fraction X lose radio-activity, and these only if the pulse was as short as 5 minutes.
13. The incorporation of labelled leucine into the cell fractions of the segments 0 - 1.6, 1.6 - 3.4 and 3.4 - 6.4 mm has been compared. There are some difference, e.g. the ribosomes from older segments lose more of their radioactivity more quickly during the chase.
14. The pattern of incorporation into root tips which have been cultured in sucrose differs from that into the controls. The uptake of labelled leucine into the supernatant is greater in cultured than in fresh root segments. As a result of this the cell fractions continue to accumulate radioactivity over a longer period.

15. The effect of actinomycin D on the incorporation of leucine into protein is to depress, but not to inhibit it.

XI. REFERENCES.

Acs, G. Reich, E. & Valansu, S. (1963) Biochim. biophys. Acta 76, 68 - 79 RNA metabolism of B. subtilis: effects of actinomycin.

Allen, J.M. & Gockerman, J. (1964) Ann. N.Y. Acad. Sci. 121 Art. 2 616-633. Electrophoretic separation of multiple forms of particle associated acid phosphatase.

Allfrey, V.G., Daly, M.M. & Mirsky, A.E. (1953) J. Gen. Physiol. 37 157 - 175 Synthesis of protein in the pancreas II The role of ribonucleoprotein in protein synthesis.

Allfrey, V.G., Hopkins, J.W., Frenster J.H. & Mirsky, A.E. (1960) Ann. N.Y. Acad. Sci. 88 Art. 3 722 - 740. Reactions governing incorporation of amino acids into the proteins of the isolated cell nucleus.

André, J. & Marinozzi, V. (1965) J. de Microscopie 4 615 - 626. Presence, dans les mitochondries, de particules ressemblant aux ribosomes.

Appel, S.H., Alpers, D.H. & Tomkins, G.M. (1965) J. Mol. Biol. 11 12 - 22. Multiple molecular forms of β - galactosidase.

Bal, A.K. & Gross, P. (1963) Science 139 584 - 586. Mitosis and differentiation in roots treated with actinomycin.

Blank, F. & Frey-Wyssling, A. (1944) Ann. Bot. 8 71 - 78. Protoplasmic growth in the hypanthium of Oenothera acaulis during cell elongation.

Boardman, N.K. & Partridge, S.M. (1955) Biochem. J. 59 543 - 552. Separation of neutral proteins on ion-exchange resins.

Bonner, J., Huang, R.C., & Gilden, R.V. (1963) Proc. Nat. Acad. Sci., Wash. 50 893 - 899. Chromosomally directed protein synthesis.

Borsook, H., Deasy, C.L., Hagen-Smit, A.J., & Keighley, G. (1950) J. Biol. Chem. 184 529 - 543. The uptake in vitro of ^{14}C - labelled glycine, L - leucine, and L - lysine by different components of guinea pig liver homogenate.

Brachet, J. (1954) Nature 174 876 - 877. Effects of ribonuclease on the metabolism of living root tip cells.

Brachet, J. & Denis, H. (1963) Nature 198 205 - 206. Effects of actinomycin D. on morphogenisis.

Brown, R. & Rickless, P. (1949) Proc. Roy. Soc. B 136 110. A new method for the study of cell division and cell extension with some preliminary observations on the effect of temperature and nutrient.

Brown, R. (1963) Symp. Soc. exp. Biol. 17 1 - 17. Cellular differentiation in the root.

Brown, R. & Broadbent, D. (1950) J. exp. Bot. 1 248 - 263. The development of cells in the growing zones of the root.

Brown, R. & Cartwright, P.M. (1953) J. exp. Bot. 4 197. Proteolytic enzymes in growing root cells.

Brown, R. & Robinson, E. (1955) In 'Biological Specificity and Growth'. Ed. E.G. Butler. Princetown University Press.

Brown, R. & Sutcliffe, J.F. (1950) J. exp. Bot. 1 88 - 113. The effects of sugar and potassium on extension growth in roots.

Bucknall, R.A. & Sutcliffe, J.F. (1965) J. exp. Bot. 16 587 - 604. The nucleic acids and cellular differentiation in the root apex of Pisum sativum II The nucleic acids and cellular development patterns.

Cahn, R.D., Kaplan, N.O., Levine, L. & Zwilling, E. (1962) Science 136 962 - 966. Nature and development of lactic dehydrogenases.

Chang, L.O., Srb, A.M., & Steward, F.C. (1962) Nature 193 756 - 759. Electrophoretic separations of the soluble proteins of Neurospora.

Cherry, J.H. & Hageman, R.H. (1961) Plant Physiol. 36 163 - 168. Nucleotide and ribonucleic acid metabolism of corn seedlings.

Consden, R., Gordon, A.H. & Martin, A.J.P. (1946) Biochem. J. 40 33 - 41. Ionophoresis in silica jelly: a method for the separation of amino acids and peptides.

Coolidge, T.B. (1939) J. Biol. Chem. 127 551 - 553. A simple cataphoresis apparatus.

Cooper, A.H., Harris, G. & Neal, G. (1962) Biochim, biophys. Acta 61 573 - 582. The incorporation of labelled amino acids into subcellular fractions of brewers' yeast.

Cruft, H.J. (1962) Biochem. J. 84 47 p. The electrophoresis of histones in polyacrylamide gels.

Das, H.K. & Roy, S.C. (1961) Biochim, biophys. Acta 53 445 - 446. Protein synthesis in plant mitochondria.

Davies, D.D. (1961) Biochem. J. 80 93 - 99. The specificity of malic dehydrogenase from higher plants.

Derrien, Y. (1952) Biochim. biophys. Acta 8 631 - 640. Studies on proteins by means of salting-out curves. I Method of establishment of salting-out curves of proteins.

Dorner, R.W., Kahn, A. & Wildman, S.G. (1957). Plant Phsiol. 32 (xii) Proc. of Plant Physiol. meetings. The soluble protein composition of expanding and ageing tobacco leaves.

Fox, D.J., Thurman, D.A. & Boulter, D. (1963) Biochem. J. 87 29 p. Electrophoresis of plant proteins in polyacrylamide gels.

Gordon, A.H., Keil, B., & Sebesta, K. (1949) Nature 164 498 - 499. Electrophoresis of proteins in agar jelly.

Grogg, E., & Pearce, A.G.E. (1952) J. Path. & Bact. 64 627 - 636. A critical study of the histochemical techniques for acid phosphatase with a description of an azo-dye method.

Haldar, D., Freeman, K., & Work, T.S. (1966) Nature 211 9 - 12. Biogenisis of mitochondria.

Hellebust, J.A. & Bidwell, R.G.S. (1964) Can. J. Bot. 42 1 - 12. Protein turnover in attached wheat and tobacco leaves.

Heyes, J.K. (1960) Proc. Roy. Soc. B. 152 218 - 230. Nucleic acids changes during cell expansion in the root.

Holmes, B.E., Mee, L.K., Hornsey, S. & Gray, L.H. (1955) Exp. Cell. Res. 8 101 - 113. The nucleic acid content of cells in the meristematic, elongating and fully elongated segments of roots of Vicia faba.

Huang, R.C. & Bonner, J. (1962) Proc. Nat. Acad. Sci., Wash., 48 1216 - 1222. Histone, a supressor of chromosomal ribonucleic acid (RNA) synthesis.

Hultin, T. (1961) Exp. Cell. Res. 25 405 - 417. Activation of ribosomes in sea urchin eggs in response to fertilization.

Hurwitz, J., Furth, J.J., Malamy, M. & Alexander M. (1962) Proc. Nat. Acad. Sci., Wash., 48 1222. The role of deoxyribonucleic acid in ribonucleic acid synthesis III The inhibition of the enzymatic synthesis of ribonucleic acid and deoxy ribonucleic acid by actinomycin D and proflavin.

Jacob, F. & Monod, J. (1961) Cold Spring Harbor Symp. on Quantitative Biol. 26 193 - 209. On the regulation of gene activity.

Jensen, W.A. (1955) Exp. Cell. Res. 8 506 - 522. A morphological and biochemical analysis of the early phases of cellular growth in the root tip of vicia faba.

Jensen, W.A. (1958) Exp. Cell. Res. 14 575 - 583. The nucleic acid and protein content of root tip cells of Vicia faba and Allium cepa.

Jensen, W.A. & Ashton, M. (1960) Plant Physiol. 35 313 - 323. The composition of the developing primary wall in onion root tips.

Johnson, B.C. & Hall, O. (1965) Amer. J. Bot. 52 506 - 513. Analysis of phylogenetic affinities in the Triticinae by protein electrophoresis.

Kates, J.R. & Goldstein, L. (1964) J. Protozoo. 11 30 - 35. A comparison of the protein composition of three species of amoebae.

Keller, E.B., Zamecnik, P.C. & Loftfield, R.B. (1954) J. histochem. cytochem. 2 378 - 386. The role of microsomes in the incorporation of amino acids into proteins.

Kirk, J.M. (1960) Biochim, biophys. Acta 42 167 - 169. The mode of action of actinomycin D.

Kohn, J. (1958) Clin. Chim. Acta 3 450 - 454. Small scale membrane filter electrophoresis and immuno-electrophoresis.

Kunkel, H.G. & Slater, R.J. (1952) Proc. Soc. exptl. Biol. Med. 80 42 - 44. Zone electrophoresis in a starch supporting medium.

Kunkel, H.G. & Tiselius, A. (1952) J. Gen. Physiol. 35 89 - 118. Electrophoresis of protein on filter paper.

Laties, G.G. & Budd, K. (1964) Proc. nat. Acad. Sci., Wash. 52 462 - 469. The development of differential permeability in isolated steles of corn roots.

Levinthal, C., Keynan, A. & Higa, A. (1962) Proc. nat. Acad. Sci., Wash. 48 1631 - 1638. Messenger RNA turnover and protein synthesis in B. Subtiles inhibited by Actinomycin D.

Levinthal, C., Signer, E.R. & Fetherolf, K. (1962) Proc. nat. Acad. Sci., Wash. 48 1230 - 1237. Reactivation and hybridization of reduced phosphatase.

Loening, U.E. (1961) Biochem. J. 81 254 - 260. Changes in microsomal components accompanying cell differentiation of pea-seedling roots.

Loening, U.E. (1965) Biochem. J. 97 125 - 133. Synthesis of messenger ribonucleic acid in excised pea-seedling root segments. Separation of the messenger from microsomes by electrophoresis.

Lowry, O.H., Rosebrough, N.J., Farr, A.C. & Randall, R.J. (1951) J. Biol. Chem. 193 265 - 275. Protein measurement with the Folin phenol reagent.

Luck, D.J.C. & Reich, E. (1964) Proc. nat. Acad. Sci., Wash. 52 931 - 938. DNA in mitochondria of Neurospora crassa.

Lund, H.A., Vatter, A.E. & Hanson, J.B. (1958) J. biophys. biochem. Cytol. 4 87 - 97. Biochemical and cytological changes accompanying growth and differentiation in the root of Zea mays.

Lyndon, R.F. (1963) J. exp. Bot. 14 419 - 430. Changes in the nucleus during cellular development in the pea seedling.

MacDonald, I.R., Knight, A.H. & De Kock, P.C. (1961) Physiol. Plant. 14 7 - 19. Metabolic processes associated with growth in storage tissue discs.

Mans, R.J. & Novelli, G.D. (1961) Arch. biochem. biophys. 94 48 - 53. Measurement of the incorporation of radioactive amino acids into protein by a filter paper disc method.

Markert, C.L. & Moller, F. (1959) Proc. nat. Acad. Sci., Wash. 45 753 - 763. Multiple forms of enzymes: Tissue, Ontogenetic, and species specific patterns.

McCure, D.C. (1961) Ann. N.Y. Acad. Sci. 94 Art. 3 723 - 730. Multiple peroxidases in corn.

McKinlee-McKee, J.S. & Moss, D.W. (1965) Biochem. J. 94 583 - 587. Heterogeneity of liver alcohol dehydrogenase on starch gel electrophoresis.

Mertz, D. (1961) Americ. J. Bot. 48 405 - 413. Distribution and localization of ascorbic acid oxidase in the maize root tip.

Monod, J., Changeux, J.P. & Jacob, F. (1963) J. Mol. Biol. 6 306 - 329. Allosteric proteins and cellular control systems.

Morgan, C. & Reith, W.S. (1954) J. exp. Bot. 5 119 - 135. The composition and quantitative relations of protein and related fractions in developing root cells.

Moore, R.O. & Villee, C.A. (1963) Science 142 389 - 390. L-malate dehydrogenase: multiple forms in separated blastomeres of sea urchin embryos.

Mornis, R.O. (1966) Biochim, biophys. Acta 127 273 - 276. Changes in pea root proteins associated with tissue differentiation: the effect of 2, 4 -dichlorophenoxy - acetic acid.

Ornstein, L. & Davis, B.J. (1959) J. Histochem. cytochem. 7 291. Simultaneous preservation of intracellular phorphological and enzymatic or antigenic activities in frozen tissues for high resolution histo chemistry.

Ornstein, L. & Davis, B.J. (1961) Preprint distributed by Distillation Products Industries Division, Eastman Kodak Co. Rochester, N.Y. Disc electrophoresis.

Ornstein L. (1964) Ann. N.Y. Acad, Sci. 121 Article 2. p. 321 - 349. Disc electrophoresis. I Background and theory.

Oster, G. (1954) Nature 173 300 -301. Dye sensitized photopolymerization of acrylamide.

Oudin, J. (1947) Bull. Soc. Chim. Biol. 29 140 - 149. L'analyse immunochimique du sérum de cheval por précipitation specifique en milieu gélifié. Premiers resultats.

Ouchterlony, O. (1949) Ark. Kemi. Mineral. och Geol. 268 No. 14 1 - 9. Antigen - antibody reactions in gels.

Parthier, B. (1963) Biochim. biophys. Acta 72 503 - 505. Mitochondria as important sites of protein synthesis in the cells of green leaves.

Paul, J. & Struthers, M.G. (1963) Biochem. Biophys. Res. Comm. 11 135 - 139. Actinomycin D - resistant RNA synthesis in animal cells.

Perry, R.P. (1963) National Cancer Inst. Monograph No. 14 73 - 87. Role of the nucleolus in ribonucleic acid metabolism and other cellular processes.

Peterson, E.A. & Sober, H.A. (1956) J. Amer. Chem. Soc. 78 751 - 755. Chromatography of proteins I Cellulose ion-exchange absorbents.

Picton, H. & Linder, S.E. (1897) J. Chem. Soc. 71 568. Solution and pseudosolution. Part III The electrical convection of certain dissolved substances.

Poblick, M.D. (1957) Nature 180 1477 - 1479. Starch gel electrophoresis in a discontinuous system of buffers.

Racusen, D. & Calvanico, N. (1964) Anal. Biochem. 7 62 - 66. Preparative electrophoresis on polyacrylamide gel.

Raymond, S. (1964) Ann. N.Y. Scad. Sci. 121 Art. 2 350 - 364. Acrylamide gel electrophoresis.

Raymond, S. & Nakamichi, M. (1962) Anal. Biochem. 3 23 - 30. Electrophoresis in synthetic gels. I Relation of gel structure to resolution.

Raymond, S. & Nakamichi, M. (1964) Anal. Biochem. 7 225 - 232. Electrophoresis in synthetic gels. II The effect of gel concentration.

Raymond, S. & Wang, Y. (1960) Anal. Biochem. 1 391 - 396. Preparation and properties of acrylamide gel for use in electrophoresis.

Raymond, S. & Weintraub, L. (1959) Science 130 711. Acrylamide gel as a supporting medium for zone electrophoresis.

Reich E., Franklin, R.M., Shatkin, A.J. & Tatum, E.L. (1961) Science 134 556 - 557. The effect of actinomycin D on cellular nucleic acid synthesis and virus production.

Reich, E., Goldberg., I.H. & Rabinowitz, M. (1962) Nature 196 743 - 748. Structure-activity correlations of actinomycins and their derivatives.

Reis, P.J., Coote, J.L. & Work, J.S. (1959) Nature 184 165 - 167. Protein biosynthesis and oxidative phosphorylation in isolated rat liver mitochondria.

Reisfeld, R.A., Lewis, U.J. & Williams, D.E. (1962) Nature 195 281- 283. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels.

Rendi, R. (1959) Exptl. Cell. Res. 17 585 - 587. On the occurrence of intramitochondrial mucleoprotein particles.

Richards, E.G. & Gratzer, W.B. (1964) Nature 204 878 - 879. An investigation of soluble RNA by zone electrophoresis.

Robertson, A.I. (1966) Ph.D. Thesis of Edinburgh University. Metabolic changes during callus development in tissue isolated from Jerusalem Artichoke tubers.

Robinson, E. (1956) J. exp. Bot. 7 296 - 305. Proteolytic enzymes in growing root cells.

Robinson, E. & Brown, R. (1952) J. exp. Bot. 3 356 - 374. The development of the enzyme complement in growing root cells.

Robinson, E. & Brown, R. (1954) J. exp. Bot. 5 71 - 78. Enzyme changes in relation to cell growth in excised root tissues.

Robinson, E. & Cartwright, P.M. (1958) J. exp. Bot. 9 430 - 435. Nucleolytic enzymes in growing root cells.

Roodyn, D.B., Reis, P.J. & Work, T.S. (1961) Biochem. J. 80 9 - 21. Protein synthesis in mitochondria: requirements for the incorporation of radioactive amino acids into mitochondrial proteins.

Sawaki, S., Morikawa, N. & Yamada, K. (1965) Nature 207 523 - 524. Malic dehydrogenase isozymes.

Shatkin, A.J. (1962) Biochim. biophys. Acta 61 310 - 313. Actinomycin inhibition of ribonucleic acid synthesis and poliovirus infection of HeLa cells.

Smithies, O. (1955) Biochem. J. 61 629 - 641. Zone electrophoresis in starch gels: group variations in the serum of normal human adults.

Sober, H.A., Gutter, F.J., Wyckoff, M.M. & Peterson, E.A. (1956) J. Amer. Chem. Soc. 78 756 - 763. Chromatography of proteins II Fractionation of serum protein on onion-exchange cellulose.

Sober, H.A. & Peterson, E.A. (1954) J. Amer. Chem. Soc. 76 1711 - 1712. Chromatography of proteins on cellulose ion-exchangers.

Spiegel, M., Ozaki, H. & Tyler, A. (1955) Biochem. Biophys. res. Comm. 21 135. Electrophoretic examination of soluble proteins synthesized in early sea urchin development.

Stedman, E. & Stedman, E. (1950) Nature 166 780 - 781. Cell specificity of histones.

Stevenson, M.L., Thimann, K.V. & Zamecnik, P.C. (1956) Arch. Biochem. Biophys. 65 194 - 209. Incorporation of ^{14}C - amino acids into proteins of leaf discs and cell free fractions of tobacco leaves.

Steward, F.C. & Bidwell, R.G.S. (1966) J. exp. Bot. 17 726 - 741. Storage pools and turnover systems in growing and non-growing cells: experiments with ^{14}C-suerose, ^{14}C-glutamine and ^{14}C-asparagine.

Steward, F.C., Bidwell, R.G.S. & Yemm, E.W. (1956) Nature 178 734. Protein metabolism, respiration and growth. A synthesis of results from the use of ^{14}C-labelled substrates and tissue cultures.

Steward, F.C. (1958) Amer. J. Bot. 45 709. Growth and organized development of cultured cells. III Interpretations of the growth from free cells to carrot plants.

Steward, F.C. & Barber, J.T. (1964) Ann. N.Y. Acad. Sci. 121 Art. 2 525 - 531. The use of acrylamide gel electrophoresis in the investigation of the soluble proteins of plants.

Steward, F.C., Lyndon, R.F. & Barber, J.T. (1965) Amer. J. Bot. 52 155- 164. Acrylamide gel electrophoresis of soluble plant proteins: a study of pea seedlings in relation to development.

Steyne-Parve, E.P. & van den Hout, A.J. (1953) Biochim. biophys. Acta 10 320 - 325. On the variability of the salting-out curves of proteins of normal human plasma and serum.

Sundaram, T.K. & Fincham, J.R.S. (1964) J. Mol. Biol. 10 423 - 437. A mutant enzyme in Neurospora crassa interconvertible between electrophoretically distinct active and inactive forms.

Sutcliffe, J.F., Bollard, E.G. & Steward, F.C. (1960) J. exp. Bot. 11 151 - 166. The incorporation of ^{14}C into the proteins of particles isolated from plant cells.

Synge, R.L.M. & Tiselius, A. (1950) Biochem. J. 46 xLi. Fractionation of hydrolysis products of amylose by electrokinetic ultra filtration in an agar-agar jelly.

Thorne, C.J.R., Grossman, L.I. & Kaplan, N.O. (1963). Biochim. biophys. Acta 73 193 - 203. Starch gel electrophoresis of malate dehydrogenase.

Thurman, D.A., Palin, C. & Laycock, M.V. (1965) Nature 207 193 - 4. Isoenzymatic nature of L - glutamic dehydrogenase of higher plants.

Ting, I.P., Sherman, I.W. & Dugger, W.M. Jr. (1966) Plant. Physiol. 41 1083 - 1084. Intracellular location and possible function of malic dehydrogenase isozymes from young maize root tissue.

Tiselius, A. (1937) Biochem. J. 31 1464 - 1477. Electrophoresis of serum globulin. II Electrophoretic analysis of normal and immune sera.

Tiselius, A.,(Hjerten, S. & Levin, O" (1956) Arch. Biochem. Biophys. 65 132 - 155. Protein chromatography on calcium phosphate columns.

Trakatellis, A.C., Axelrod, A.E., & Monkjar, M. (1964) Nature 203 1134 - 1136. Actinomycin D and messenger RNA turnover.

Ts'O, P.O.P. (1962) Ann. Rev. Plant. Physiol. 13 45 - 80. The ribosomes - nucleoprotein particles.

Ts'O, P.O.P. & Sato, C.S. (1959) J. Biochem. Biophys. Cytol. 5 59 - 68. The incorporation of leucine ^{14}C into microsomal particles and other subcellular components of the pea epicotyl.

Vaughan, D. (1965) Ph.D. Thesis of Edinburgh University. The effects of purine and pyrimidine analogues and related substances on extension growth.

Warner, J.R., Knopf, P.M. & Rich, A.R. (1963) Proc. nat. Acad. Sci., Wash. 49 122 - 130. A multiple ribosome structure in protein synthesis.

Watson, J.D. (1963) Science 140 17 - 26 Involvement of RNA in the synthesis of proteins.

Webster, G.C. (1954) Plant Physiol. 29 202 - 203. An anergy dependent incorporation of amino acids into the protein of plant mitochondria.

Wettstein, F.O., Staehelin, T. & Noll, H. (1963) Nature 197 430 - 435. Ribosomal aggregate engaged in protein synthesis characterization of the ergosome.

Woodward, D. & Munkres, K.D. (1966) Proc. nat. Acad. Sci., Wash. 55 872 - 880. Alterations of a maternally inherited mitochondrial structural protein in respiratory deficient strains of Neurospora.

Wright, S.T.C. (1960) Nature 185 82 - 85. Occurrence of an organ specific antigen associated with the microsome fraction of plant cells and its possible significance in the process of cellular differentiation.

Wright, S.T.C. (1963) Symp. Soc. exptl. Biol. 17 18 - 39. Cellular differentiation at the molecular level with special reference to proteins.

Wroblewski, F. (1961) Ann. N.Y. Acad. Sci. 94 Art. 3. Multiple molecular forms of enzymes. (Conference editor)

Young, R.W. & Fulhorst, N.W. (1965). Anal. Biochem. 11 389 - 391. Recovery of ^{35}S radioactivity from protein-bearing polyacrylamide gel.

Also;

Brown, R + Wightman, F. (1952) J. Exp. Bot. 3 253-263. The influence of mature tissue on division in the meristem of the root.

Caspersson, T.O. (1947) Symp. Soc. Exp. Biol. 1 127-151. The relations between nucleic acid and protein synthesis.

Cook, F.S. (1959) Can. J. Bot. 37 621-639. Generative cycles of protein and trasaminase in the growing corn radicle.

ACKNOWLEDGEMENTS.

I should like to express my gratitude to my supervisor, Dr. U.E. Loening, for his helpful advice throughout the course of this work. My thanks are due also to Professor R. Brown, F.R.S., for his interest, to other members of the Department of Botany for helpful discussions and to Mrs J. Newman for the preparation of the typescript.

The grant from the Department of Scientific and Industrial Research (later the Science Research Council) is gratefully acknowledged.

XIII. APPENDIX.

(i) Abbreviations.

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
NAD	nicotinamide adenine dinucleotide
PPO	2, 5 - diphenyl oxazole
POPOP	1, 4 - bis (5 - phenoxazol - 2 - yl) benzene
TEMED	N,N,N',N' - tetramethylethylenediamine
TRIS	2 - amino - 2 (hydroxy - methyl) - propane - 1:3 diol

(ii) Sources of Chemical.

Chemicals were of analytical grade and obtained from British Drug Houses except for these listed below.

Acrylamide	Kodak, Liverpool.
N,N-methylenebisacrylamide	L. Light and Co. Ltd., Colinbrook, England.
N,N,N',N'-tetramethylethylene diamine	L. Light and Co. Ltd., Colinbrook, England.
N-methyl phenazonium methosulphate	L. Light and Co. Ltd., Colinbrook, England.
Amido Black 10B Michrome brand	Edward, Gurr, 42 Upper Richmond Road, London, E.C.1.
Nitro blue tetrazolium	Edward Gurr, 42 Upper Richmond Road, London, E.C.1.
Bromophenol blue Nivoc indicator	W. & J. George and Becker Ltd., London, E.C.1. and Birmingham 3.
Naphthol AS acetate	K & K Rare Chemicals Kodak, Liverpool.
α - naphthyl phosphoric acid	K & K, Rare Chemicals " "
o - anisidine tetrazolium salt	K & K Rare Chemicals " "
Diazo blue B	K & K Rare Chemicals " "
Araldite	CIBA
PPO) POPOP)	Packard Instrument Co., Chicago
Actinomycin D	gift from Merck Sharp and Dohme

^{14}C-leucine was obtained from the Radiochemical Centre, Amersham, Bucks. For first experiments, DL-leucine-^{14}C (72mC/mM) was used: for later experiments L-leucine-^{14}C (160 mC/mM) was used.

The labelled leucine used for each experiment was a large enough quantity for it to be cleaned by chromatography on a Dowex column and re-used:-

The Dowex 50 resin was cleaned and regenerated by cycling with 1N HCl, water, 1N NaOH and water. It was then packed into a column of 1 cm. diam. and 3 cm in length. The leucine was applied to the column in water and the column washed with water and HCl of increasing concentrations from 0.1 N - 2N, before the leucine was removed with NH_4OH, 10% and then 20%, the latter bringing out the bulk of the leucine. The solution was evaporated and the leucine redissolved in water.